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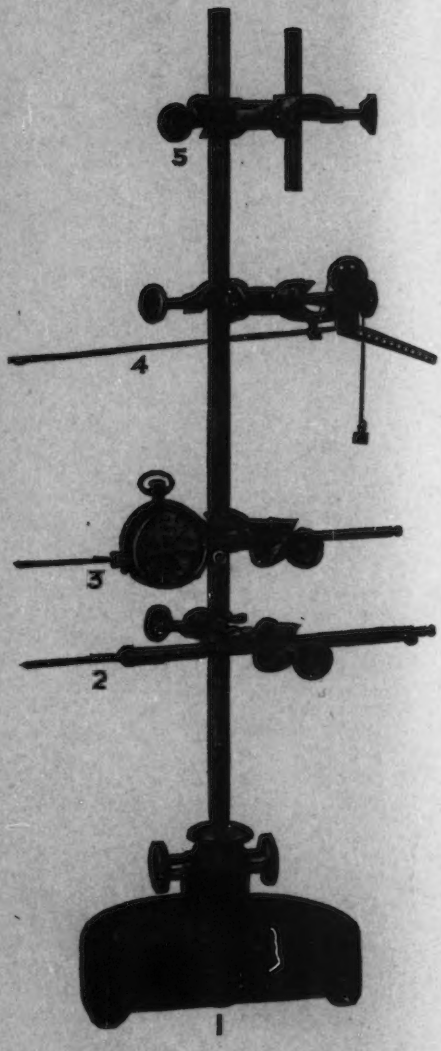

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THE DETERMINATION OF RATE OF HEMOLYSIS BY THE MEASUREMENT OF LIGHT TRANSMISSION

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The literature on the properties of the red blood corpuscle in regard to its stability is profuse. The impression is obtained, however, that the currently accepted views on these properties are not always based on data and procedures of an accuracy which warrant some of the conclusions drawn. Our object in this work was primarily to ascertain whether certain of these views are based on physical measurements of sufficient accuracy for the purposes intended. The present paper deals with the method adopted for the study of the rate of hemolysis.

PREVIOUS METHODS. The rate at which red blood corpuscles disintegrate under the action of a hemolytic agent can be studied in several ways. A frequently used method is that of determining the time necessary for complete hemolysis to occur. Numerous end-points for this have been suggested, including complete transparency, and the reading of print through the hemolyzing suspension. Such end-points are necessarily subjective, and but one point in the process is observed. One further finds partial hemolysis observations reported as "slight hemolysis," "marked," etc. Such data must obviously be merely qualitative.

A second general method consists in removing samples from the hemolyzing mixture at intervals, and determining the amount of hemolysis in each sample by red blood cell count, or by attempting to stop the hemolysis by chilling or centrifuging, and determining the amount of hemoglobin liberated. This method is open to criticism in that hemolysis cannot be completely arrested instantaneously, and only the slower hemolytic processes can be followed.

A third method attempts to determine the amount of hemolysis from

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moment to moment, by following the variations in transmission of energy, such as heat or light, through the red cell suspension. Thus Noyons (1) passed a beam of light through a hemolyzing mixture and allowed it to strike a thermopile. He compared the current induced in the thermopile by this beam with the current induced in an identical thermopile by a standard beam. The results were expressed only in per cent of absorption of the light by the unknown compared with a blood suspension of known strength or with a smoked glass. He also tried a selenium cell but found its action too lagging and unsteady.

Ponder (2) used a Crookes vane radiometer to measure the emergent beam, noting the period of revolution of the radiometer at frequent intervals. He translated these periods into percentage of hemolysis by comparing them with the known periods of revolution of the same radiometer under the influence of beams which had passed through suspensions containing known percentages of hemolyzed and unhemolyzed cells. More recently (3) Ponder has also used a selenium cell to measure the intensity of the emergent beam.

Jacobs (4) has followed hemolysis by placing the red cell suspension before a carbon filament lamp, and varying the thickness of the suspension until the filament was just visible. This is open to the same criticism as other subjective end-points, although its simplicity is attractive.

PRESENT METHOD. In the method presented in this paper, a beam of light was passed through a dilute blood suspension to which a hemolytic agent had been added. The emergent beam was allowed to fall on a potassium hydride photo-electric cell.[2] The current thus set up was recorded at frequent intervals, and by suitable calibration of the apparatus, this current was translated into percentage hemolysis.

DESCRIPTION OF APPARATUS. A beam of light composed of relatively parallel rays was obtained by using a Bausch and Lomb microscope light equipped with a 108 watt, 6 volt, ribbon filament, Mazda projection lamp. This lamp was operated on a storage battery, with the voltage across the lamp terminals maintained constant by a sliding contact rheostat of heavy nickel wire. As a further precaution to insure constancy not only of the source of light, but of the apparatus as a whole, at frequent intervals during an experiment the beam of light was passed through the same spot in a piece of gold red plate glass of approximately the same light absorbing power as the blood suspension studied. The current through the lamp was then adjusted if necessary until the photo-electric cell current was the same as at the beginning of the experiment, or the small change in current was noted,

[2] The photo-electric cell was kindly furnished through Dr. H. B. Williams of the Department of Physiology, College of Physicians and Surgeons, Columbia University, by Dr. H. E. Ives and Mr. H. C. Snook of the Bell Telephone Laboratories, New York, to whom we are gratefully indebted.

and suitable corrections were calculated for the readings obtained with the blood suspension. After traversing a two-inch water chamber to absorb heat rays, the beam of light was passed through the dilute blood suspension under investigation. This was contained in a cylindrical glass chamber, of an approximate volume of 40 cc., having plane, parallel glass sides, about one inch apart. This chamber was placed with its flat sides vertical, and normal to the light beam, and by suitable motor-driven apparatus, it was kept slowly revolving between readings to keep the cells uniformly distributed. Provision was made for a constant temperature water or air bath around the glass chamber, but subsequent observations demonstrated that the small variations in room temperature (between 0.5 and 2°C.) which occurred during an experiment produced a negligible effect on the

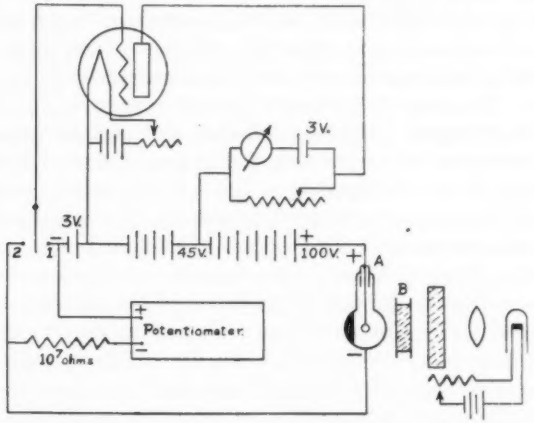

Fig. 1. Photo-electric cell connections: A, photo-electric cell. B, blood suspension.

rate of hemolysis. The emergent beam passed through a small circular aperture, and impinged on the photo-electric cell, which was mounted in an otherwise light-tight box. A shutter on the lamp shielded the blood suspension and the photo-electric cell from the light beam between readings.

In the dark, a photo-electric cell transmits an inappreciable current, but as light strikes it, electrons pass from the negatively charged potassium hydride film to the anode. The amount of this current, in a properly made cell, is directly proportional to the intensity of illumination, provided the composition of the light beam remains constant. The response of the cell increases, in general, as the frequency of the incident light increases. Such a cell possesses the advantages of high sensitivity to minute variations in intensity of the incident beam, and also of practically instantaneous

response and recovery. One is able, accordingly, to determine at very frequent intervals the amount of light traversing the blood suspension.

The apparatus used for the measurement of the photo-electric cell current in this work is diagrammed in its main features in figure 1.[3] Into the plate circuit of a vacuum tube is introduced a shunt circuit of two branches, one consisting of a variable resistance, the other of a galvanometer and a source of e.m.f. This shunt circuit functions as a simple potentiometer circuit, and by suitable adjustment of the variable resistance, the current flowing through the galvanometer is reduced to approximately zero. The grid of the vacuum tube is connected to a single pole, double throw switch. When this switch is closed in position one, the grid is connected to a biasing battery. The grid is, accordingly, at a definite negative potential with respect to the filament, and a certain plate current is flowing. The other contact of the double throw switch is connected to the negative pole of the photo-electric cell, and when the switch is closed in position two, the grid potential is changed from that of the biasing battery to that of this negative pole. This, in general, changes the plate current, and the galvanometer needle is deflected. The photo-electric cell current passes through a high fixed resistance, with a resultant fall in potential, or IR drop. This fall in potential is now balanced by a Leeds and Northrup potentiometer in series, until the galvanometer needle returns to its original position indicating that the original grid potential and the original plate current have been restored. The potentiometer reading, after deduction of a zero correction, gives a reading directly proportional to the intensity of the light striking the cell. The zero correction is the potentiometer reading when no light strikes the photo-electric cell, and is due chiefly to ionization in the vacuum tube, together with leakage through the base, and the photo-electric cell dark current. This correction is reduced to a minimum by careful selection of a relatively gas-free and well insulated tube, and by using a low plate voltage.

Two points may be noted with regard to the arrangement of the apparatus. The potentiometer is so placed that its readings are uninfluenced by the vacuum tube characteristics. A further important fact is that the galvanometer is placed in the vacuum tube plate circuit, where a small change in the photo-electric cell negative pole potential and therefore in grid potential produces a marked change in plate current and hence in galvanometer deflection. This makes possible the use of a much less sensitive galvanometer than would be necessary were it placed directly in the photo-electric cell circuit.

The values indicated in figure 1 were found to be convenient, and using

[3] We wish to express our deep appreciation to Prof. H. W. Webb and Mr. D. R. White of the Department of Physics, Columbia University, for invaluable aid and suggestions concerning the electrical apparatus, and the arrangement of it.

them a fall in potential of one-half millivolt across the high fixed resistance could be measured. This corresponded to a photo-electric cell current of approximately 0.5×10^{-10} amperes.

When the light transmission of a blood suspension was being studied, the voltage across the electric lamp was adjusted to the strength of the blood suspension, so that a suitable photo-electric cell current was obtained with no hemolysis. Additional range was obtained by varying the voltage applied to the photo-electric cell terminals, and by the use of the proper factor, an IR drop measured at one photo-electric cell terminal voltage could be converted into the corresponding IR drop at a second voltage. This principle was used in almost every hemolysis experiment, because the photo-electric cell current, when of suitable magnitude at the lower percentage of hemolysis, became too large to handle on the potentiometer at the higher percentages. The current also became dangerously large for transmission through the photo-electric cell and the high fixed resistance. In the apparatus as arranged, a reduction in photo-electric cell terminal voltage from approximately 100 to 30 volts reduced the photo-electric cell current and hence the fall in potential across the high fixed resistance to about $\frac{1}{12}$ of their former values.

SPONTANEOUS CHANGES IN LIGHT TRANSMISSION. Early in the work certain spontaneous changes were noted in the light transmission through dilute blood suspensions. Until a method was devised to obviate these changes, it was out of the question either to calibrate the apparatus or to follow a hemolytic process. It was found that if freshly drawn, citrated or oxalated, human blood, or washed cells from such blood, were diluted in various isotonic fluids, and allowed to stand 15 to 20 minutes, the light transmission as determined at that time and again at the end of 1 to 3 hours frequently had changed markedly (5). The change was usually a diminution in light transmission. This occurred in about half of the suspensions, irrespective of diluent, and was of a magnitude of from 5 to 40 per cent of the original light transmission. Occasionally an increase in light transmission was noted. In the range of blood concentration studied, i.e., 0.25 to 1.0 per cent, no constant differences due to varying concentration could be detected. Various diluting fluids were used to attempt to prevent these changes, but without success. These included *1*, 0.85 per cent sodium chloride; *2*, a modified Ringer's solution; *3*, an isotonic phosphate-buffered NaCl solution, containing 0.01 per cent of $CaCl_2$ and having a pH of 7.3; *4*, Locke's solution with gelatin and sucrose as described by Rous (6), and *5*, a solution devised by Brinkman (7) and recommended by Ponder (8) containing 0.7 per cent NaCl, 0.1 per cent KCl, 0.2 per cent $NaHCO_3$, and 0.02 per cent $CaCl_2$. This solution as originally described was to be equilibrated with carbon dioxide to give it a pH of 7.35. The equilibrated solution was found difficult to work with and no more satisfactory than the

non-equilibrated. Without CO_2 the solution yielded a fine precipitate of calcium carbonate on standing 12 hours. To obviate this, two other solutions were used, both similar to the non-equilibrated Brinkman's solution, except that one contained only 0.1 per cent of $NaHCO_3$, the other only 0.01 per cent of $CaCl_2$. No correlation between the changes in light transmission and the pH of the diluting fluid, or of the outside fluid at the end of an experiment could be determined.

The shape of the red cells was studied on flamed glass slides. The findings of Brinkman and Van Dam (9) and of McGlone (10) were definitely confirmed to the effect that biconcave red cells in an isotonic diluting fluid rapidly crenate and soon become spherical when placed on a glass slide which has been polished by rubbing. When such a rubbed slide is flamed or allowed to stand for some time before use, no such changes in cell shape are produced. In the case of the cells in the various diluting fluids used, no correlation could be demonstrated between changes in cell shape, and in light transmission. Irregular forms of crenation were customarily observed.

The following technique was eventually developed to obtain a red cell suspension of constant light transmission. Washed cells to represent 0.25–0.5 per cent whole blood were added to a diluting solution similar to Brinkman's solution, having the following composition: NaCl 0.7 per cent, KCl 0.1 per cent, $NaHCO_3$ 0.1 per cent, anhydrous $CaCl_2$ 0.02 per cent, (pH 7.9). Such blood suspensions were allowed to stand 18 to 24 hours in paraffined flasks. Reasonable precautions against bacterial contamination were taken, but it was found that after standing for such a period at room temperature, the suspensions frequently showed small amounts of hemolysis, and often bacteria could be demonstrated in them. These suspensions had, however, reached a point of relative constancy as regards light transmission. Identical suspensions, allowed to stand at 2 to 4°C. for 18 to 24 hours, and then left at room temperature for 2 to 3 hours, also were relatively constant as regards light transmission, with hemolysis varying between 0 and 1 per cent. Similar suspensions, made up in isotonic NaCl, and allowed to stand for such a period at either room temperature or on ice, showed a change in light transmission in about one-third of the cases. Cells which had reached an apparent equilibrium with the outside diluting fluid had assumed a spherical shape, crenated with numerous small, delicate, point-like projections. Those in saline, on the other hand, were usually coarsely and irregularly crenated. The irregular currents or streaming seen in freshly diluted blood when shaken disappeared as equilibrium was reached. Apparently the calcium content of the solution is one factor in the production of equilibrium. When the calcium content was reduced to one-fourth of the usual amount, the cells did not reach equilibrium on

standing, were irregularly crenated, and there was present the streaming seen in freshly diluted blood.[4]

CALIBRATION OF APPARATUS. The apparatus was calibrated by determining the magnitude of the photo-electric cell current, i.e., the fall in potential across the high fixed resistance as measured on the potentiometer, produced by the beam of light which had passed through a series of standard suspensions made up to represent 0, 10, 20, 40, 60, 80, 90 and 100 per cent hemolysis. These were freshly made up for each experiment by hemolyzing the proper amounts of concentrated red cell suspension in 24 cc. portions of distilled water, and allowing to stand one hour to insure maximum stromatolysis. The hemolysates were rendered isotonic by adding 25 cc. of diluting solution of twice the above concentration. Precipitation of calcium carbonate was avoided by adding the calcium chloride separately, dissolved in 1 cc. of water. The respective balance of intact red cells was then added to each to yield the proper final concentration, which as a rule represented 0.4 per cent blood. These suspensions were allowed to stand in paraffined flasks at 2 to 4°C. for 18 to 20 hours, rapidly restored to room temperature, shaken occasionally during the following 2 to 3 hours, and the light transmission through each determined.

The question arose as to whether it was correct to calibrate the apparatus using standard suspensions made up by distilled water hemolysis, and then use the calibration curve so obtained in interpreting light transmission through saponin hemolysates. Distilled water usually leaves intact many red blood corpuscle shadows or ghosts.[5] Saponin destroys them, presumably increasing the light transmission. This was studied by using two identical glass chambers, each optically equivalent to half the glass chamber as ordinarily used to contain the blood suspension. The two parts of a given standard suspension made up as described above, i.e., one-half containing only hemolyzed cells, the other only unhemolyzed cells, were then placed without mixing into the two half chambers, and the light transmission through the pair determined. Saponin was then added to the hemolyzed blood fraction, and the light transmission through the pair redetermined. It was found that, using 0.4 per cent blood, the increase in light transmission with the addition of saponin was usually negligible until 100 per cent hemolysis was reached. At this point the addition of saponin increased the light transmission sufficiently to represent 0.5 to 1.0

[4] The above technique refers to human red blood corpuscles. Dog corpuscles hemolyze extensively when allowed to stand several hours in a paraffined flask at 2 to 4°C. Rabbit corpuscles, on the other hand, can be handled similarly to human corpuscles. They do not, however, reach as complete an equilibrium as human cells.

[5] This statement refers to human blood. In similar concentration, rabbit corpuscles are completely broken up by distilled water, so that subsequent addition of saponin does not increase the light transmission.

per cent hemolysis, i.e., the light transmission at 100 per cent hemolysis without saponin represented 99.5 to 99 per cent hemolysis with saponin. Occasionally a difference equivalent to 0.5 per cent hemolysis was noted in the case of the 90 per cent hemolysis standard, but this was uncommon. Even using 0.25 per cent blood, an error representing 0.5 per cent hemolysis in the range between 95 per cent and 100 per cent hemolysis was not infrequent. Accordingly, in obtaining a calibration curve, the standard

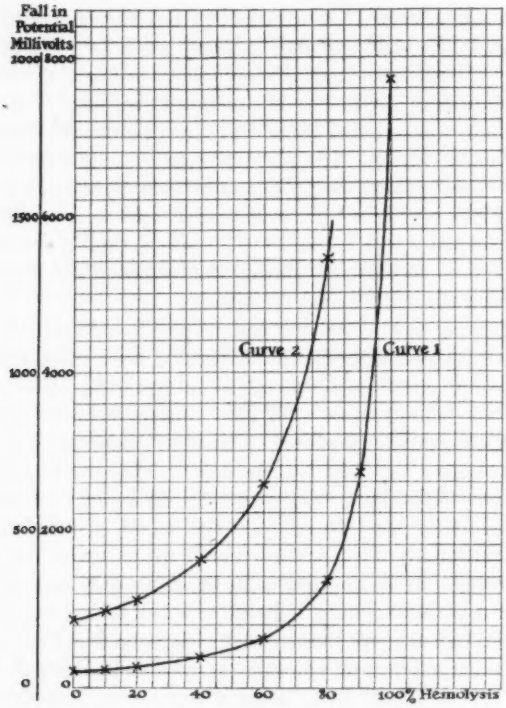

Fig. 2. Calibration curve. To facilitate reading, the lower portion of curve *1* has been replotted with the ordinates increased fourfold (curve *2*).

suspension representing 100 per cent hemolysis was treated with saponin in excess before determining its light transmission. A typical calibration curve appears in figure 2 for a series of standard suspensions as described above, with complete stromatolysis produced in the one representing 100 per cent hemolysis. Abscissae represent per cent of hemolysis. Ordinates represent light transmission through blood suspensions each containing a proper proportion of hemolyzed and unhemolyzed cells, and contained

within the full size glass chamber. No simple mathematical expression has been determined for this curve. It approaches a translated hyperbola.

TECHNIQUE OF HEMOLYSIS EXPERIMENT. The calibration curve for a given experiment having been obtained, a red cell suspension identical with the calibration suspension containing no hemolyzed cells and similarly brought to equilibrium, was centrifuged. Half the volume of the supernatant fluid was removed, and the desired amount of hemolytic agent added to it. If saponin was used, it was added in about 1.0 cc. of diluting solution, and an equivalent volume of the supernatant fluid was discarded. The cells were resuspended in the other half of the supernatant fluid, and the two rapidly mixed from two burettes of equal rate of outflow. The suspension was immediately transferred to the glass chamber, and readings of the light transmission were made as frequently as indicated, in some cases every half

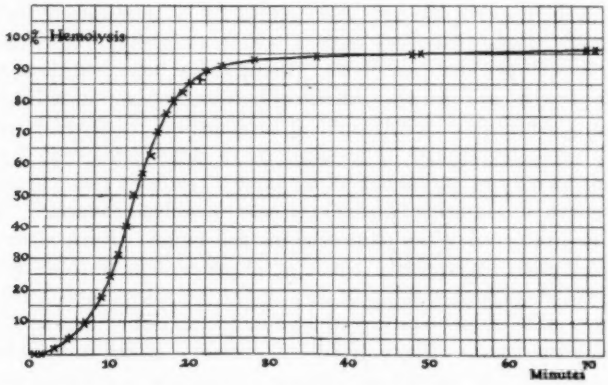

Fig. 3. Hemolysis time curve (saponin 1:90,000)

minute, with constant slow revolution of the glass chamber between readings. The amount of light transmission as recorded by the photo-electric cell was then converted into per cent of hemolysis by reference to the calibration curve, and a curve obtained such as figure 3 which is a typical curve representing the hemolysis of a 0.4 per cent blood suspension under the action of saponin in 1:90,000 dilution. It will be noted that there is a brief lag period before hemolysis begins. It then proceeds at a progressively faster rate until approximately 40 per cent of hemolysis has been reached, and then gradually slows up, yielding an asymmetrical sigmoid curve. A few red blood corpuscles can usually be seen under the microscope after several hours. No "ghosts" are seen. This time curve has an approximate accuracy at any point along its course of $\pm$ 0.5 per cent of hemolysis. Further experimental curves will be given in a subsequent report.

COMMENT. Certain observations are perhaps of interest in comparing the above work with that of Ponder. In the main the experimental time curves agree, although Ponder apparently found it unnecessary to let the blood suspensions stand to reach a state of equilibrium. He described his standards as made up from finger capillary blood, whereas his hemolysis experiments were evidently done on suspensions made up with washed cells from citrated venous blood. Numerous workers (11), (12), (13) have found differences in the red cell count and hemoglobin content of capillary and venous blood. Also even traces of blood plasma tend to protect the red cells from crenation, and to buffer the suspensions (14). These factors alter the light transmission of blood suspensions in varying degrees.

As noted by Ponder (3) the vane radiometer is more sensitive to small differences in percentage hemolysis in the lower ranges of hemolysis, while the selenium cell, like the potassium hydride photo-electric cell, is more sensitive in the upper. We are unable, however, to corroborate the uppermost portion of the hemolysis curves, which Ponder described as rising promptly to 100 per cent hemolysis. Using blood concentrations ranging from 0.25 per cent to 1 per cent, all the cells were not hemolyzed even after 2 to 3 hours, unless a concentration of saponin was used which produced extremely rapid hemolysis. The usual curve sloped off gradually above 90 per cent hemolysis, and blood counts done on samples taken at points on the time curve representing 95 to 99 per cent hemolysis invariably checked within $\frac{1}{4}$ to $\frac{1}{2}$ per cent of the photo-electric cell figure. Even after several hours in the usual experiment, (reaching 90 per cent in 20 to 30 minutes), a few red blood corpuscles could be seen under the microscope. We are, in general, not inclined to place any great reliance on cell counts as a method of following the course of hemolysis, both because of the well-known considerable error of blood counts, and because during the progress of hemolysis at a significant rate it is obviously impossible to count. In the present case, however, the presence of intact cells shows conclusively that hemolysis is not complete.

SUMMARY

A method is described for the determination of rate of hemolysis, using a photo-electric cell to follow changes in light transmission from moment to moment through the hemolyzing suspension.

Certain spontaneous changes in light transmission through dilute blood suspensions are discussed.

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A STUDY OF SAPONIN HEMOLYSIS OF NORMAL HUMAN BLOOD WITH SOME OBSERVATIONS ON ANEMIA BLOOD

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There has been described in a previous paper (1) a method for the determination of the rate of hemolysis, based upon the measurement of the intensity of a beam of light which has traversed the blood suspension. The present paper deals with the rate of hemolysis by saponin of various bloods, as determined by this method.

TECHNIQUE. The technique used in the determination of the rate of hemolysis consisted, briefly, in adding saponin to a dilute suspension of washed red blood cells in a buffered isotonic solution (1) containing NaCl, KCl, $NaHCO_3$, and $CaCl_2$. The suspension, previous to the addition of the saponin, had been permitted to reach a state of relative equilibrium as regards light transmission. A beam of light was passed through the hemolyzing mixture, and allowed to fall on a photo-electric cell. The changing current so set up was measured at frequent intervals, and by suitable calibration it was interpreted as percent of hemolysis. With exceptions as noted, standard conditions of temperature, cell, and saponin concentrations were maintained throughout the work.

NORMAL HUMAN BLOOD. Figure 1 contains the hemolysis curves of several bloods taken from as many healthy, supposedly normal, persons. Ordinates represent percent of hemolysis, abscissae time in minutes since the addition of saponin. The red blood cell concentration was uniform, 2×10^7 cells per cubic centimeter of hemolyzing mixture, or 0.4 per cent of a blood containing 5,000,000 cells per $mm.^3$ The temperature varied between 20° and 22°C., a negligible range as indicated below. The saponin used in all determinations was obtained from a single bottle of Eimer & Amend saponin, marked "White. Pure." A fresh solution of it was prepared for each day's determinations, and allowed to stand 24 hours before being used, as it was found that very fresh solutions sometimes yielded irregular results. It was used in a concentration of 1:100,000. All determinations were made in duplicate, but for the sake of clearness in the figure, only the mean of each pair was reproduced, with a single exception.

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The deviation between the two determinations of any given pair was of the order of magnitude represented in the pair marked "Duplicate." Both curves of this typical pair have been reproduced, together with the observed points used in plotting them. The actual observed data for the two curves are given in table 1. A large majority of the observed points used in plotting any given curve were directly on the line of the curve. The remainder were within a range of ± 1 per cent of hemolysis.

The curves from these several normals have the same general sigmoid shape with, however, a certain degree of variation. Since this variation is not markedly greater than the deviation between duplicate determinations made at the same time, and since repeated determinations over several months on the same individual frequently showed approximately

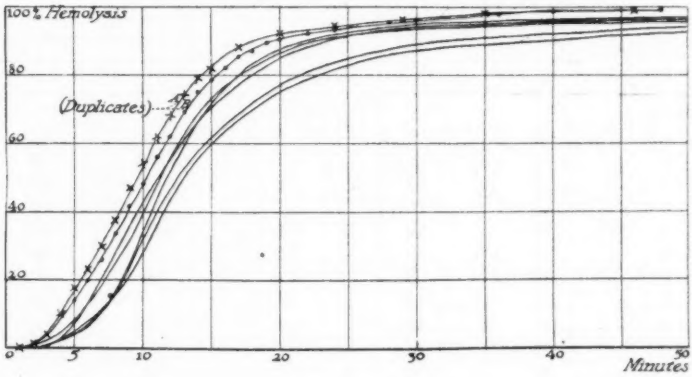

Fig. 1. Hemolysis time curves of normal human blood. Saponin 1:100,000. Curves A and B represent a typical pair of curves from duplicate determinations. Each of the other curves represents a different individual.

an equal amount of variation, it is reasonable to look on it as due either to uncontrolled alterations in experimental conditions, or to changes in the bloods.

EFFECT OF CHANGE OF TEMPERATURE. In the study of the rate of saponin hemolysis, at least three variables require control. These are temperature, red cell concentration, and saponin concentration. It is generally agreed that increase in temperature reduces the time of hemolysis by any particular concentration of saponin. Ponder (2) found the curve representing this relationship to be a hyperbola, approaching the temperature axis as one asymptote. In the concentration of saponin used in the work reported in this paper, variations in temperature between 20° and 25°C. were found to produce deviations in results within experimental

error. Presumably the curve showing the relationship between temperature and time of hemolysis was approximately parallel to the temperature axis in this range.

EFFECT OF CHANGE IN RED BLOOD CELL CONCENTRATION. The effect of change in the concentration of red blood cells on the rate of hemolysis was studied in a considerable series of determinations. Simultaneous determinations of the rate of hemolysis were made in duplicate on blood suspensions of two or more different strengths using the same concentration of saponin. The ratio between the periods, expressed in minutes, required to attain a certain per cent of hemolysis of the two red blood cell concentrations was determined. The power to which it was necessary to raise this ratio

TABLE 1

Experimental data for hemolysis time curves A and B, figure 1

TIME AFTER ADDITION OF SAPONIN	PERCENTAGE HEMOLYSIS		TIME AFTER ADDITION OF SAPONIN	PERCENTAGE HEMOLYSIS	
	A	B		A	B
minutes			*minutes*		
1	0	0	16		82
2	1	1	17	88	85
3	4	4	18		87
4	10	10	19		89
5	18	14	20	92	91
6	23	20	22		92
7	30	26	24	94	93
8	38	34	28		95
9	47	42	29	96	
10	54	48	30		96
11	62	56	35	98	
12	68	62	36		98
13	74	69	40		98½
14	79	75	46	99	
15	82	79	48		99

between the two concentrations of red blood cells for it to equal the time ratio was then calculated. The essential data of these experiments are given in table 2 (A). The exponent was found to be approximately one. In other words, the rate of hemolysis is inversely proportional to the cell concentration.

EFFECT OF CHANGE IN CONCENTRATION OF SAPONIN. The effect of saponin concentration on the rate of hemolysis is somewhat more complex. A group of experiments was carried out in an attempt to evaluate this. With a constant red cell concentration and temperature, simultaneous determinations were made of the rate of hemolysis, using two or more different concentrations of saponin. The ratio of the number of minutes required to

attain a certain per cent of hemolysis was determined. The power to which it was necessary to raise this ratio between the two saponin concentrations for it to equal the time ratio was calculated. The data of these

TABLE 2

Relation of erythrocyte concentration and of saponin concentration to time required to attain various degrees of hemolysis

A. RELATION OF RED CELL CONCENTRATION TO TIME					B. RELATION OF SAPONIN CONCENTRATION TO TIME				
Experiment	$\frac{\text{Conc. r.b.c}}{\text{Conc.}_1 \text{ r.b.c}}$	Percentage hemolysis	$\frac{\text{Time}}{\text{Time}_1}$	Exponent*	Experiment	$\frac{\text{Conc.}_1 \text{ sap.}}{\text{Conc. sap.}}$	Percentage hemolysis	$\frac{\text{Time}}{\text{Time}_1}$	Exponent†
			minutes					*minutes*	
2–14	2.0	30	10/6½	0.6	3–9	2.0	30	11/2½	2.1
		50	13/8½	0.7			50	17/3½	2.3
		80	22/13	0.8			80	35/5	2.8
		90	36/17	1.1			90	56/6½	3.1
3–9	2.0	30	11/8	0.5	4–4	1.25	30	8½/5½	2.0
		50	17/10½	0.7			50	10½/7	1.8
		80	35/17½	1.0			80	15/9½	1.9
		90	60/30	1.0			90	19/11½	2.3
4–4	1.17	30	6/5	1.2	4–8	1.25	30	10/6½	1.9
4–8		50	7/6	1.0			50	12/8	1.8
		80	9½/8½	0.7			80	17/11	1.8
		90	11½/10	0.9			90	22/14	2.0
		30	8½/7½	0.8	4–10	1.25	30	7½/5	1.8
		50	10/9	0.7			50	9/6	1.8
		80	15/13	0.9			80	13/8½	2.1
		90	19/16	1.1			90	16/10	2.1
	1.33	30	6½/5	0.9		1.67	30	14/5	2.0
		50	8/6	1.0			50	17/6	2.0
		80	11/8½	0.9			80	27/8½	2.3
		90	14/10	1.2			90	38/10½	2.5
		30	9½/7½	0.8	4–19	1.25	30	8½/5½	2.0
		50	12/9	1.0			50	11/6½	2.4
		80	17/13	0.9			80	18/10	2.6
		90	22/16	1.1			90	23/12	2.9
Mean of exponents.....				0.9 ± 0.2					

* The exponent represents the power to which it is necessary to raise the ratio of erythrocyte concentrations for it to equal the time ratio.

† The exponent represents the power to which it is necessary to raise the ratio of saponin concentrations for it to equal the time ratio.

determinations are given in table 2 (B). It will be noted that this exponent was approximately two during about the first half of hemolysis, and it then tended to increase, so that a given change in concentration of saponin produced a greater effect on the rate of hemolysis. During the

greater portion of the reaction, the rate of hemolysis appears to be directly proportional to the square of the saponin concentration.

HUMAN ANEMIAS. A few determinations were made of the rate of saponin hemolysis of red blood cells from patients with secondary anemias (fig. 2). The anemias were due to repeated hemorrhages from duodenal or gastric ulcers, and were moderately severe, the hemoglobin being less than 50 per cent of normal, the red cell count about 50 per cent of the normal, with a reticulocyte percentage of less than 1 per cent. Red cell suspensions were made up of the same strength, i.e., 2×10^7 cells per cubic centimeter, and in the same manner as the normal bloods. Temperature and saponin concentration were identical to those used with normal bloods. The rate of hemolysis of these cells was somewhat slower than

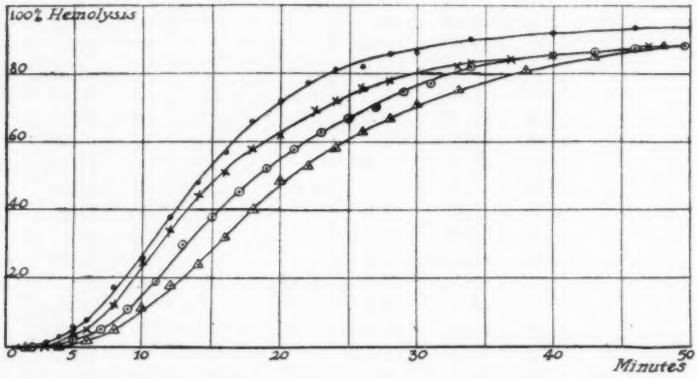

Fig. 2. Hemolysis time curves of anemic human blood. Saponin 1:100,000. The curve having the observed points indicated by × is from a case of pernicious anemia. The others are from three cases of secondary anemia.

the normals, although not markedly so. The same general type of reaction is manifested. When cell volume, instead of cell number, was used as a basis for making up the suspensions, the cell number and surface were increased, inasmuch as the red cells in secondary anemia are smaller than normal. This resulted in a somewhat slower rate of hemolysis. A determination was also made of the rate of hemolysis of a single pernicious anemia blood, having a count of 1,000,000 red cells per mm.^{3} hemoglobin of 20 per cent, and a typical blood picture. It did not differ from the secondary anemia group (fig. 2).

While these few determinations of the rate of hemolysis of anemia bloods by saponin are without particular significance, the observations of several other workers may be mentioned. Bigland (3) reported an increased resistance to saponin hemolysis over that of normal blood in all types of

anemia except pernicious anemia. Neilson and Wheelon (4), using sapotoxin, found an increased resistance in anemias associated with pulmonary tuberculosis, acute infections, carcinoma, hemorrhage, lead poisoning, obstructive jaundice, and some cases of pernicious anemia, but a decreased resistance in lues and hemolytic jaundice. M'Neil (5), on the contrary, reported a decrease in resistance in secondary anemias and pernicious anemia. What data we have agree with the findings of Bigland, and of Neilson and Wheelon.

To throw further light on the question of resistance to saponin of cells from an anemic animal, rabbits were rendered anemic by bleeding. The determinations of the rate of hemolysis of the red blood cells from these animals will be reported in a subsequent paper.

SUMMARY

1. A group of curves is presented recording the rate of saponin hemolysis of red blood cells of normal individuals.

2. A few determinations were made of the rate of saponin hemolysis of blood from persons with severe secondary anemias. These cells hemolyzed somewhat slower than the normals.

3. The effects of change of temperature, red cell concentration, and saponin concentration are discussed.

We are indebted to the staff of the Presbyterian Hospital, New York City, for access to the patients whose blood was utilized in the above work.

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A STUDY OF SAPONIN HEMOLYSIS OF RETICULOCYTE-CONTAINING BLOOD

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The curve representing the rate at which red blood cells are hemolyzed by saponin is sigmoid in form, as indicated in figure 1, A. If one determines the slope of such a curve at various points along its course, plots these slopes as ordinates at their proper positions in the time axis, and draws a smooth curve through the points so located (1), a derivative curve is obtained, represented at B, figure 1. Ponder (2), Brooks (3) and others have explained the general shape of the derivative curve as an expression of the distribution of resistance of the red cells to hemolysis. According to this view, the cell resistance is distributed approximately symmetrically about a mode.

It was thought that perhaps some evidence for or against the existence of such a distribution of cell resistance could be obtained by inducing a severe anemia in an animal, and studying the blood so obtained. If resistance is a function of age, it seemed reasonable that a blood consisting of relatively young cells should hemolyze at a different rate than normal blood, and that the derivative curve should give some indication of the change in distribution of resistance. M'Neil (4) found an increased rate of saponin hemolysis in blood from individuals with secondary anemia. Bigland (5) reported the reverse, i.e., a decreased rate of hemolysis or an increased resistance to saponin in secondary anemia. Neilson and Wheelon (6), using sapotoxin, reported an increased resistance in most types of secondary anemia. Handovsky (7) found the red cells from rabbits in which an anemia had been induced by bleeding, to be first less resistant and then more resistant to saponin than normal. By diluting such blood with saline, and centrifuging, he was able to separate the more resistant cells from the less resistant, the former remaining suspended in the upper portion of the tube, the latter going to the bottom. Key (8) reviewed the evidence for considering reticulocytes as young cells, a generally accepted view, and noted that when blood containing reticulocytes was allowed

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to stand, or was slowly centrifuged, the reticulocytes tended to separate out in the upper part of the cell layer.

In the work described in this paper, determinations were made of the rate of saponin hemolysis of the red cells from normal rabbit blood, and from the blood of the same animal after a severe secondary anemia had been rapidly induced by bleeding. The anemia blood cells were studied as obtained from the blood, and also after they had been separated by sedimentation into a high and a low reticulocyte fraction. The reticulocytes were determined in the usual way, by staining with brilliant cresyl blue, making a smear, and counter-staining with Wright's blood stain. Five hundred or 1000 red cells were counted, depending on the concentration of reticulated cells. The anemias were induced by removing from 25 to 35

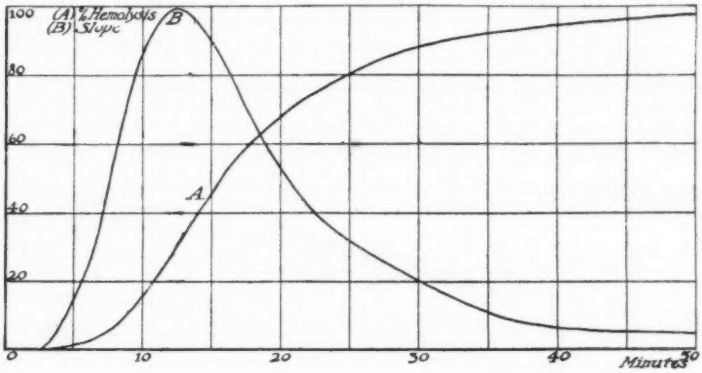

Fig. 1. Hemolysis time curve and derivative. A—Time curve. B—Derivative. The maximum slope of the time curve has been taken as 100, and the slope at other points has been plotted to this scale.

cc. of blood daily by cardiac puncture. From three to five such bleedings were required to reduce the red cell count 50 per cent or more, and to increase the reticulocyte percentage from that normally found in the average laboratory rabbit, 0.5 to 2 per cent, up to around 20 per cent. Using the technique for the determination of rate of hemolysis as described in a previous paper (9), determinations were done on the blood obtained in the first bleeding. This provided a normal for the animal. When a suitable reticulocyte count had been attained, the animal was exsanguinated from a carotid artery. A sample was removed from this blood, handled as usual, and determinations made of the rate of saponin hemolysis. The remainder of the blood was separated into a low and a high reticulocyte fraction, either by allowing to stand in the cold for a few hours, or by slow

centrifuging, the cells of each fraction washed, suspended and equilibrated to constant light transmission as usual, and determinations were then made of the rate of saponin hemolysis. Temperatures were relatively

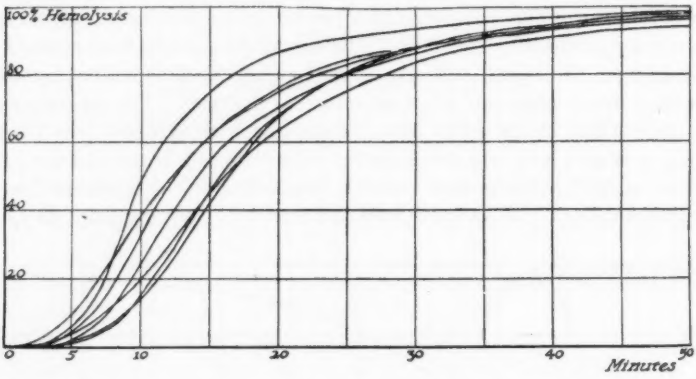

Fig. 2. Hemolysis time curves of normal rabbit blood. Saponin 1:167,000

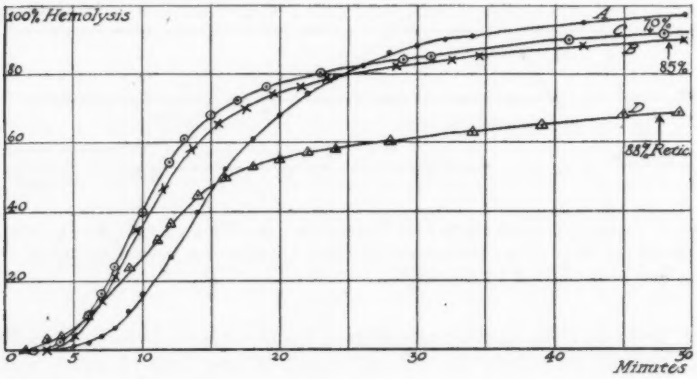

Fig. 3. Hemolysis time curves of anemic rabbit blood. Saponin 1:167,000. A—Cells from original normal blood. B—Cells from anemia blood before separation (reticulocytes 23 per cent). C—Low reticulocyte fraction (11 per cent). D—High reticulocyte fraction (53 per cent).

Arrows with accompanying figures indicate points at which reticulocyte counts were made on the remaining cells, with the values found.

constant throughout, between 20° and 22°C. The saponin used was from a single bottle, the same as that employed in previous experiments (labelled "Saponin, Eimer & Amend, White, Pure"). A fresh solution was made up

for each experiment, and allowed to stand over-night before use. A concentration of 1:167,000 was employed in all experiments, i.e., 0.24 mgm. of saponin per 40 cc. of red cell suspension. A uniform concentration of red cells was maintained in each experiment, 2×10^7 per cubic centimeter of hemolyzing suspension. Determinations were invariably made in duplicate, but to avoid confusion only the mean of each pair has been reproduced. Duplicate curves regularly had the same general shape, being relatively parallel, with a difference between the two almost always less than one minute for the first 50 per cent of hemolysis, gradually increasing beyond this but usually less than three minutes at 90 per cent hemolysis.

Figure 2 contains the curves representing the rate of hemolysis by saponin, under standard conditions, of the red blood cells from seven normal rabbits. Five of these animals survived the repeated bleedings and the blood from each was used as described above. In figure 3 is given the complete set of curves from a typical experiment, including the normal for the same animal. At the beginning of the experiment, this rabbit had a red blood cell count of 5,100,000 per mm.^{3}, with 1.2 per cent of reticulocytes. He was bled four consecutive days, a total of 135 cc. of blood being removed. At the end of this period, he was exsanguinated, and the blood so obtained had a red cell count of 1,300,000 per mm.^{3}, with 23 per cent reticulocytes. By sedimentation, a low reticulocyte and a high reticulocyte fraction were obtained, containing respectively 11 per cent and 53 per cent reticulocytes.

It will be noted from figure 3 that the cells from the whole anemia blood, from the low reticulocyte fraction, and from the high reticulocyte fraction, all hemolyze somewhat more rapidly in the first portion of the reaction than the cells from the original normal blood. The higher the reticulocyte count, however, the earlier did the reaction slow up until hemolysis became definitely slower than in the normal. The results in all the experiments were essentially similar to this. The one selected shows, if anything, somewhat less difference from the normal than others. In one experiment, the high reticulocyte fraction (66 per cent) had reached only 50 per cent hemolysis at the end of 1½ hours.

Pepper and Peet (10) called attention to the danger of error in counting reticulocytes following partial hemolysis by hypotonic saline. The reticulum tends to remain intact after the cell has hemolyzed. By counterstaining with Wright's blood stain, however, the presence or absence of hemoglobin-containing protoplasm can be definitely determined. Such counts were made on the hemolyzing suspensions at the points indicated by arrows on the curves, and the percentage of reticulocytes was noted. From these results it is apparent that the non-reticulated or older cells hemolyze first, with resulting concentration of the reticulocytes. Even

in the low reticulocyte fraction an initial reticulocyte percentage of 11 had increased to 70 at the end of fifty minutes. It seems reasonable to conclude that the reticulocytes or young cells are definitely more resistant to saponin than the older or than the normal cells.

Figure 4 represents the derivatives of the set of curves of figure 3, obtained as described above, i.e., by measuring the slope of the time curve at numerous points, plotting these slopes as ordinates at their respective points on the time axis, and drawing a curve through the tops of the ordinates. From these derivative curves, one notes that during the first

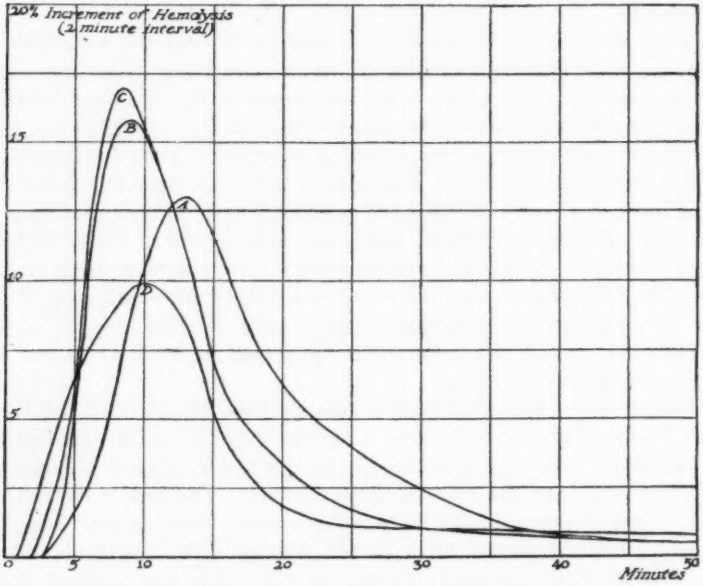

Fig. 4. Derivatives of hemolysis time curves of figure 3

ten minutes of hemolysis, the anemia blood and both fractions of it hemolyzed at a greater rate than the normal blood. The point of maximum rate is reached in each case at approximately the same time, and earlier than in the normal. There is no evidence of an increased tendency of the cells to hemolyze at one time, as would be expected if the younger cells, being more nearly of an age than normal cells, possessed a more uniform resistance to saponin than normal. (Even doubling the concentration of the saponin in the case of the high reticulocyte fraction did not change the general shape of the reaction curve, merely speeding it up.) On the contrary, in the high reticulocyte fraction, and accordingly the fraction con-

taining the greatest number of young cells, the derivative curve, if it represents resistance, shows a spreading. From the reticulocyte counts made during the hemolysis, it is clear that the cells falling in the region of the mode of the derivative curve, i.e., the cells which would have the more uniform resistance, are non-reticulocytes. The reticulocytes are largely represented in the farther and lower portion of the curve, the hemolysis of but comparatively few cells having occurred in any given time interval. On a resistance basis, accordingly, the reticulocytes possess the more varying resistances. At the same time one must conclude that the rate of hemolysis of reticulocytes is slower than of normal blood. Whether this is due to a real increase in resistance in the reticulocytes, or to some other factor, such as increased inhibition of hemolysis by the products liberated from previously hemolyzed cells, remains to be seen.

In studying the degree of hemolysis produced in blood of hibernating bats by varying amounts of saponin, Handovsky (7) obtained curves which would yield bimodal derivative curves. This, if substantiated, would be good evidence indeed for the theory that the derivative curve represents resistance distribution about a mode. If there is any fairly homogeneous group of cells entering the blood stream during regeneration, one would expect the time curve of saponin hemolysis of such blood, at least of the high reticulocyte fraction, to be bimodal also. The absence of such a bimodal feature seems to us to detract definitely from the evidence for the above interpretation.

Discussion. *Relation of resistance to age of cells.* Snapper (11), Hamburger (12), and others have shown that resistance to hemolysis by hypotonic salt solution increases when the proportion of young cells in the blood has been increased due to regeneration after bleeding. Having shown that the younger cells may be more resistant they conclude that in general the older cells form the more labile and the younger cells the more stable groups. Handovsky's (7) results (saponin concentration curve) as well as the saponin time curves given above show that more resistant cells do appear in the blood during rapid regeneration, but if age of the cells were the determining factor, the rate curve, i.e., the derivative of the time curve, could never be bell-shaped, but would necessarily be at a maximum at the end corresponding to the youngest cells. If one still wishes to attribute a general rôle to age of cells, one is obliged to make the additional assumption that a large group of middle-aged cells are fairly homogeneous in resistance and only the youngest and oldest have a markedly different resistance. This will be hard to prove or disprove and would be warranted as a fair assumption only if all other portions of the theory of hemolysis were firmly established leaving this one point as an inevitable assumption. Even then it would not carry one much further.

Incidentally it may be noted that when Hamburger (12) speaks of the

oldest cells being near the point of "vital hemolysis," there is no evidence that this "vital hemolysis" which represents the natural disintegration of the red cells has necessarily any relation to hemolysis produced by hemolytic agents such as saponin or to hemolysis by hypotonic salt solution. According to Rous (13) the usual means of red cell disintegration in the living body is by fragmentation rather than by hemolysis. Until data are available on the quantitative relation of susceptibility to fragmentation and to hemolysis it is not at all safe to assume that the life expectancy of a cell regarding fragmentation is the same as that regarding hemolysis. This is particularly true since the change in resistance of a regenerating blood to hemolysis will take opposite trends depending on whether saponin or NaOH is used (Handovsky). Another difficulty which stands in the way of establishing any simple relation between age of cells and resistance lies in the fact that during the early stages of active regeneration after bleeding the resistance may be less than normal while only later the increased resistance appears. This was shown by Handovsky in the case of saponin and is substantiated by some of our own unpublished data on resistance to hypotonic salt solution. No matter what point of view we take on the interpretation of the data, the simple assumption of Hamburger that resistance is inversely proportional to age is not substantiated.

The question of resistance distribution according to a probability curve will be dealt with in further detail in another paper. Our results so far have led us, however, to seriously question the importance attributed by some writers to this probability distribution. It is reasonable to believe that, like many chemical reactions, the hemolysis reaction is influenced by the constantly decreasing cell concentration as hemolysis proceeds. If the ordinates of a derivative curve are to represent numerically the distribution of various resistances, we believe that corrections of considerable magnitude will be necessary to take into account this decreasing cell concentration. In this case the derivative will not be a bell-shaped curve about a mode. There is on record by Yule (14) and by Robertson (15), theoretical mathematical treatment of this subject which aims at explaining the facts on a probability basis without recourse to individual cell resistance. With the coöperation of Prof. E. B. Phelps, of the Department of Sanitary Science of Columbia University, we are now reinvestigating this possibility on the basis of actual hemolysis curves.

CONCLUSIONS

1. The curve of saponin hemolysis of a reticulocyte-containing blood shows a rate of hemolysis which at first is faster and later slower than that of normal blood.

2. Rapid blood regeneration does not result in the production of even a relatively homogeneous group of more resistant cells.

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PHYSICAL DEVELOPMENT AND THE EXCRETION OF CREATINE AND CREATININE BY WOMEN

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The physiology of creatinine has challenged the interest of many investigators but, as yet, no one has been able to interpret satisfactorily the data regarding its metabolism in various conditions. Its exact function in the animal organism remains unknown. Although quantitatively it ranks second among the organic nitrogenous excretions of the body, its excretion is remarkably independent of the nitrogenous food ingested. The excretion, on the contrary, is relatively constant for each individual and is roughly proportional to the body weight or more specifically, to the amount of muscular tissue. As a rule, adult men have less adipose and more muscular tissue than adult women. The former also excrete more creatinine per unit of body weight than the latter. Thus, for men, the creatinine coefficient (expressed as milligrams of creatinine nitrogen per kilo body weight per day) ranges from 5.6 to 11.9, for women from 3.3 to 9.8 (Hunter, 1922). According to Shaffer (1908), sex, per se, has no influence.

Of equal interest is the study of the metabolism of creatine, and particularly, sexual variation as evidenced by difference in creatine excretion by adult men and women. Creatine has never been found in the urine of normal adult men (on creatine-free diets). On the other hand, it is regarded as a normal physiological constituent of the urine of adult women, although its occurrence is variable both in frequency and amount. The facts that a large proportion of the total creatine of the body occurs in muscle, that there is more creatine in striated than in smooth muscle, and that it is present to a greater degree in urines in pathological conditions involving excessive muscular catabolism, indicate that its function may be connected with muscular activity and development.

The suggestion presents itself that the difference in creatine excretion of men and women may be due to muscular rather than sexual variation, that women with well developed and well conditioned musculature may exhibit no creatinuria, that women whose creatinine metabolism is comparable to that of men may also have a similar metabolism of creatine.

This may be inferred from the theory of the saturation point of muscular tissue for creatine, indirectly from the relation between the frequency of creatinuria and the physical condition of the subjects as suggested by Stearns and Lewis (1921), and from the studies of Harding and Gaebler (1922), who postulated the constancy of the *total* creatinine coefficient in children and its similiarity to the creatinine coefficient of the adult male. The present study was undertaken to obtain experimental evidence to support or refute this suggestion.

Frequent studies of the creatinine coefficients of normal women have been reported and are well summarized by McLaughlin and Blunt (1923). The values reported range from 3.5 to 9.8 with an average figure of approximately 6.4. The recent and more extended series of observations of Rose, Ellis and Helming (1928) on a single individual show a coefficient of about 6.5.

Determinations of creatine excretion have been made less frequently and under variable conditions. Krause (1911) in studying 3 normal women, on mixed diets, found creatine excretion in 25 of 30 urines (82 per cent) in amounts ranging from "traces" to 0.11 gram per day (calculated as creatinine). In the studies of Stearns and Lewis (1921), subject S, on creatine-free diets of varying protein content, excreted creatine in amounts varying from 0.04 to 0.32 gram in 18 of the 95 twenty-four hour specimens examined (19 per cent). Subject A excreted creatine in 48 per cent of the 52 urines analyzed. Other investigators have observed creatinuria in 21 of 27 specimens (77 per cent) (Rose, Dimmitt and Bartlett, 1918), and in 13 of 14 urines (93 per cent)[1] (Rose, Ellis and Helming). McLaughlin and Blunt (1923) report that in one woman, creatine was an almost constant constituent of the day urine in amounts ranging from 0.004 to 0.024 gram per hour. Denis and Minot (1917) observed creatinuria only once in 4 urines of 2 women on a mixed diet with meat once a day, an amount of 0.24 gram. On a high protein, creatine-free diet, the same subjects excreted creatine (0.09 to 0.35 gram) on 24 of 28 days. Creatinuria disappeared on resumption of a low protein diet. Other investigators have not confirmed the variations in creatine excretion accompanying different levels of protein intake as reported by Denis and Minot.

EXPERIMENTAL. The experiments here reported were carried out to determine the creatinine excretion and the frequency of creatinuria in young women whose muscular development might be expected to be higher than that of the average woman, perhaps more nearly comparable to that of men. The subjects were 14 college girls, who were interested in pro-

[1] These figures are for the fore periods of the experiment. In the after periods, subsequent to the feeding of creatine, the excretion of creatine was very infrequent and finally ceased.

fessional training in physical education, who had a history of vigorous physical activity and who at the time of our studies had from 2 to 4 hours of such activity a day. The subjects were from 19 to 23 years of age, varied only slightly from the usually accepted standards of weight for their respective heights, and had with 2 exceptions been rated in the highest groups as far as concerns general physical condition by physicians in the annual physical examinations of the fall of the academic year 1927–1928. The two exceptions were noted as showing chronic inflammatory arthritis and functional tachycardia respectively at the time of the physical examination. In view of the supposed influence of the hyperactivity of the thyroids on creatine metabolism and the frequency of thyroid enlargement in this section, special observation was made of the occurrence of goitre in these subjects. Six of the 14 had slightly enlarged thyroids but in none was there evidence of hyperthyroidism. We feel that our group is representative of the typical young woman of a muscular development above the average.

Two 24-hour specimens of urine were collected by each subject. In view of the possible relationship between creatinuria and the phases of the sexual cycle, careful record was made of the period of collection in relation to menstrual period. Creatine and creatinine determinations were made on the same day the urine was collected according to the micromethods of Folin (1914). The picric acid used was purified according to Halverson and Bergeim (Hawk and Bergeim, 1926) and was well within the limits of purity as specified by Folin and Doisy (1916–17). It was not possible to insure creatine-free diets, but careful records of the food were obtained. The diets without exception showed little preformed creatine. Meat was ingested at only one meal of the day with one or two exceptions and in moderate or small amounts only.

The results are presented in table 1. Although the present study is not sufficiently extended to warrant definite conclusions, a comparison of the results with those of others is of interest. The creatinine coefficients are higher than those previously reported; the minimum observed in the series is 7.2, other minimum values range from 3.5 to 7.7 with an average of 5.6. The present maximum is 10.8; previous maxima range from 5.0 to 9.8 with an average of 7.8. The average of the coefficients reported here is 8.5; the averages of other investigators lie between 4.5 and 8.0, the average value being 6.4. Considering the superior physical development of the subjects of this study, the results tend to support fully the theory that urinary creatinine is independent of sexual variations per se and is an index of the proportion of muscular tissue to total body weight. The coefficients were apparently not affected by the phases of the menstrual cycle.

Examination of the frequency of the occurrence of creatinuria as previously discussed shows percentage values ranging from 19 to 93 with

TABLE 1

SUBJECT	DATE	RELATION TO MENSTRUATION*		WEIGHT	PREFORMED CREATININE	TOTAL CREATININE	CREATINE (AS CREATININE)	CREATININE COEFFICIENT
	1928	days before (−) or after (+) onset	Period	kgm.	gram	gram	gram	mgm. creatinine nitrogen per kgm. weight
R. S.	April 2	−14	I.M.	51.2	1.23	1.29	0.06	8.9
	April 16	0	M.		1.18	1.27	0.09	8.6
S. M.	May 25	−3	Pre M.	51.7	1.25	1.30	0.05	9.0
	May 28	0	M.		1.18	1.22	0.04	8.5
V. J.	April 25	+14	I.M.	55.3	1.28	1.38	0.10	8.6
	April 26	+15	I.M.		1.15	1.22	0.07	7.7
C. Mc.	April 22	−14	I.M.	55.3	1.60	1.62	0.02	10.8
	May 23	+17	I.M.		1.41	1.45	0.04	9.5
M. T.	April 25	−13	I.M.	55.8	1.28	1.33	0.05	8.6
	May 31	−8	I.M.		1.21	1.25	0.04	8.1
M. VT.	May 11	0	M.	57.6	1.14	1.14	0.00	7.4
	May 12	+1	M.		1.24	1.26	0.02	8.0
D. S.	May 25	+4	M.	57.7	1.20	1.26	0.06	7.7
	May 28	+7	Post M.		1.19	1.23	0.04	7.7
J. S.	April 1	−5	Pre M.	58.6	1.46	1.63	0.17	9.2
	April 2	−4	Pre M.		1.50	1.67	0.17	9.5
S. G.	April 5	−2	Pre M.	61.7	1.32	1.36	0.04	7.9
	April 19	+12	I.M.		1.19	1.30	0.11	7.2
M. R.	May 17	+6	Post M.	64.2	1.40	1.40	0.00	8.1
	May 18	+7	Post M.		1.47	1.48	0.01	8.5
M. H.	May 14	0	M.	64.5	1.40	1.45	0.05	8.1
	May 19	+5	M.		1.36	1.37	0.01	7.8
A. Z.	April 20	−3	Pre M.	64.9	1.73	1.86	0.13	9.9
	April 23	0	M.		1.58	1.62	0.06	9.0
A. H.	May 7	+14	I.M.	66.2	1.33	1.36	0.03	7.5
	May 9	+15	I.M.		1.55	1.60	0.05	8.7
H. B.	May 14	−6	I.M.	68.5	1.80	1.84	0.04	9.8
	May 19	−1	Pre M.		1.45	1.49	0.04	7.9
Average								8.5

* (−) and (+) represent number of days before and after onset of menstruation, respectively. (−5) to (−1) represent the premenstrual period. (0) to (+5) represent the menstrual period. (+6) to (+10) represent the postmenstrual period. (+10) to (+15) and (−6) to (−14) represent the intermenstrual period.

an average of about 65 per cent. Creatine was present in 26 of the 28 urines (93 per cent) analyzed in the present experiment. If a value less than 20 mgm. be considered as due to experimental error, the number of urines containing creatine is 24 of a total of 28 or 86 per cent. The amounts were variable, ranging from 0.17 gram to traces. It would seem, therefore, that creatinuria of women is not a consequence of poor muscular development and a lower "saturation point" of the muscles for creatine and that a higher creatinine coefficient is not associated with an absence of creatinuria.

In this limited series there seems to be no definite relationship of creatinuria to menstruation. Although we consider the number of samples from each individual too few to draw definite conclusions, the results would indicate, in support of the work of others, that there is no clearly defined relationship between creatine excretion and the various phases of the menstrual cycle.

SUMMARY

In a series of women with physical development greater than that of the ordinary woman, professional students in the courses in physical education, creatinine coefficients of the same order of magnitude as those of men were observed. The frequency of creatine excretion in the urine was similar to that usually observed in women, indicating that creatinuria in women is probably not related to the difference in muscular development between men and women.

We wish to express our appreciation of the coöperation of Dr. Margaret Bell of the Department of Physical Education for Women and of the students who acted as subjects in the experiments.

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THE EFFECTS OF PHOSPHATE BUFFERS ON INTESTINAL MOVEMENTS, AND THEIR INTERRELATION WITH CALCIUM

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Physiologic saline solutions are usually buffered by the addition of sodium bicarbonate or phosphate or both. As was shown in a preceding paper (Sollmann, von Oettingen and Ishikawa, 1928a), stabilization by bicarbonate can only be secured by adjusting its concentration to the type of aeration, which may be somewhat inconvenient. The phosphates are independent of aeration, provided only that this is sufficiently active to remove the metabolic carbonic acid. The use of phosphate, either alone or in conjunction with bicarbonate, would therefore have advantages. However, the phosphates tend to produce chemical changes with the calcium of the solutions, resulting eventually in turbidity and in precipitation of calcium as $Ca_3(PO_4)_2$ and liberation of hydrogen ions. as NaH_2PO_4 (in the simplest terms, $3\ CaCl_2 + 4\ Na_2HPO_4 = Ca_3(PO_4)_2 + 2\ NaH_2PO_4 + 6\ NaCl$). The rapidity and degree of these reactions vary with conditions, so that the changes may be large or small; and as they are irreversible, the end products depend on all the conditions to which the mixture may have been subjected, more than on the final conditions. The principles of these changes are now being studied by physicochemical methods.

Our attention was called to these complications not only by the observation of the chemical changes, but also by the functional differences in the movements of the intestines in bicarbonate and in phosphate solutions. Rona and Neukirch (1912) had already observed that the movements of Magnus segments are less regular in the phosphate solutions. The difference, however, is not merely quantitative; for while the contractions in the bicarbonated solutions are purely pendular and perfectly rhythmic, in the phosphate solutions they appear as periodic tone changes of varying and often irregular rhythms, on which the more rapid and feebler pendular contractions appear superimposed. Careful observation of the segments shows that the periodicity is really due to rapid relaxation of the pendular contraction, so that upper parts of the segment are already relaxed before

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the lower start to contract; the segment therefore does not contract as a whole, but in parts, and the periodicity is due to the superposition of these partial contractions. The "periodic" contractions, as we shall call them, can usually be distinguished readily on the tracings, by their slower rate or irregularity or both, from the pendular contractions; although in a small proportion of cases some doubt may remain.

In brief, then, the addition of sodium phosphate tends to weaken the pendular contractions and to combine the weakened contractions into periodic rhythms. The presence and degree of this functional change was found to parallel the chemical changes. With the object of investigating and explaining this correlation, the following experiments were made by the Magnus method because with this the conditions can be readily varied, and adjoining segments of intestines may be used for control. However, a further series of experiments was also made with lumen-perfusion of the entire small intestines. These will be described at the end. The Locke solution contained, per cent, NaCl = 0.9; KCl = 0.042; $CaCl_2 \cdot 2H_2O$ = 0.024; and generally dextrose = 0.1. Fifty cubic centimeters of this solution were placed in a bath, warmed to 38°, and well aerated. For control experiments the solution was buffered with 0.008 per cent of sodium bicarbonate which maintains regular activity and a nearly level pH for two or three hours (Sollmann, von Oettingen and Ishikawa, 1928b). The bicarbonate may be added in advance and the solution kept for days without change; while the phosphate buffered solutions tend gradually to become cloudy and to change in pH, as has been said; phosphated solutions

Fig. 1. Magnus segments of the small intestines of rabbits in Locke solution. Course of the changes of the contractions and pH in Locke solution buffered with 0.048 per cent sodium phosphate. The time after immersion is noted on the tracings. Time tracing in all the Magnus segments = ½ minute.

Fig. 2. Charts of change of pH. A, 0.048 per cent sodium phosphate, added in different concentrations; namely, adding for 50 cc. of the final mixture, 0.024 gram of the phosphate dissolved in: solid line, 0.1 and 1.0 cc. of water; dotted lines, 25 cc. of water. B, 0.024 per cent sodium phosphate, added as 0.1 cc. of 12 per cent per 50 cc. of Locke solution; solid line, in test tube; dotted line, Magnus experiment. C, 0.048 per cent sodium phosphate, added as 0.1 cc. of 24 per cent per 50 cc., solid line test-tube; dotted line, Magnus experiment. D, 0.008 per cent sodium bicarbonate (0.1 cc. of 4 per cent) plus 0.048 per cent sodium phosphate added as in B.

Fig. 3. Freshly prepared and old, filtered phosphated Locke solution on Magnus segments. A, at natural pH; B, adjusted to 7.8.

Fig. 4. Increased effects of increased quantities of phosphate added to Locke solution containing 0.008 per cent of sodium bicarbonate.

Fig. 5. Conversion of the periodic contractions in aged and filtered phosphated (0.048 per cent) Locke solution into the pendular contractions by the addition of 0.008 per cent sodium bicarbonate. Sodium hydroxide, B, does not effect this change.

Fig. 6. Conversion of the periodic contractions in phosphated Locke solution into pendular contractions by the addition of calcium chloride.

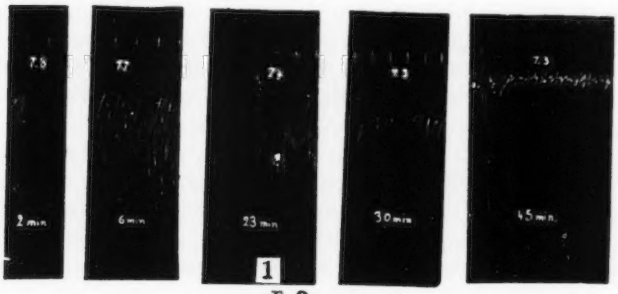
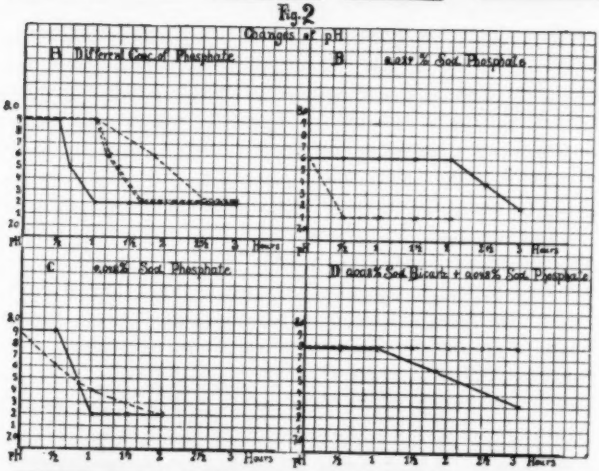
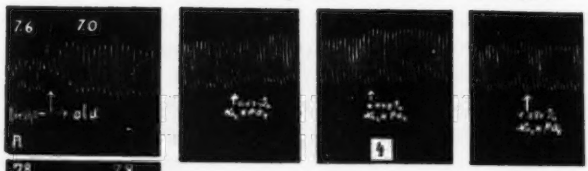

7.8
2 min.
7.7
6 min.
7.5
23 min.
1
7.3
30 min.
7.3
45 min.

Fig. 2


Changes of pH
A. Different Conc. of Phosphate
B. equiv. % Sod. Phosphate
C. .004% Sod. Phosphate
D .004% Sod. Bicarb. + .004% Sod. Phosphate
Hours


7.6
7.0
A
4


7.8
7.8
old
fresh
3


A
5


6


B

therefore need to be freshly prepared. If this is done by adding a concentrated sodium phosphate solution to the stock Locke solution, the calcium precipitation occurs more rapidly than when the phosphate is added in dilute solution, namely, by mixing equal volumes of a double-strength Locke solution and a double-strength aqueous phosphate solution. In some experiments the phosphate was added as phosphoric acid and neutralized with NaOH to the desired pH. As a rule the intestinal segments were taken from the lower end of the ileum of rabbits, shortly after death by concussion. With each procedure, a sufficient number of experiments were made, generally at least four and often more, to justify confidence in the results.

The phosphate type of intestinal contractions. If the intestinal segments are suspended in Locke solution buffered with 0.008 per cent sodium bicarbonate, they generally perform very regular pendular contractions, similar to those shown at the left of figure 1, which may continue for hours. If the Locke solution has been buffered with 0.048 per cent sodium phosphate,[2] the contractions generally start with the pendular type, but soon change progressively to the periodic type, as illustrated in figure 1. This is seen to be accompanied by increase of tone and diminished relaxation, eventually to spasticity; and is paralleled by falling of the pH from 7.8 to 7.3; the spasticity occurring between 7.4 and 7.3. The results are essentially the same whether the sodium phosphate is added as such, or formed by the neutralization of equivalent amounts of phosphoric acid (0.00134 molecular). The phenomena are also similar in 0.024 per cent of sodium phosphate, although generally less extreme, and especially with less tendency to spasm and with less fall of pH.

The changes of pH that occur when Locke solution is buffered with sodium phosphate. The addition of sodium phosphate to the Locke solution at once raises the pH to that of the phosphate, namely, to 7.8 to 8.1 for 0.048 per cent of phosphate, or to 7.5 to 7.6 for 0.024 per cent. After some time, which varies from half an hour to several days, the pH starts to fall again, rather rapidly, to about 7.2. The rapidity of the fall depends on the total concentration of the phosphate, on the temperature, and especially on the alkalinity and on the manner of mixing. It is greatest if the phosphate is added in concentrated solution to a warmed alkaline Locke solution. The changes are illustrated in figure 2. Chart A shows the important influence of the manner in which the phosphate is added, with respect to the concentration of the stock solution. In these experiments the quantity of sodium phosphate required to make a 0.048 per cent solution (0.024 gram per 50 cc.) was dissolved in 0.1, 1, 10 and 25 cc. of water, i.e., in $\frac{1}{500}$, $\frac{1}{50}$, $\frac{1}{5}$ and $\frac{1}{2}$ of the final 50 cc. volume, and added to

[2] *Throughout this paper, the quantities of "sodium phosphate" are those of the crystallized salt,* $Na_2HPO_4 . 12H_2O$.

solutions of the chlorides of the Locke mixture dissolved in 49.9, 49, 40 and 25 cc. of water. Each sample was divided into two parts, one being kept at 38°, the other at room temperature. The pH was observed colorimetrically at the periods indicated in the chart, and also after 1, 2 and 3 days. At 38°C. the concentrated solutions, in which the phosphate was dissolved in 0.1 and 1 cc. of water, gave identical results, shown by the solid line in chart A: namely, no apparent change for half an hour, then a rather abrupt fall of pH, reaching 7.2 within the hour, at which point it remained, even after several days. When the phosphate was added in dilute form, i.e., dissolved in 25 cc. of water, the fall of pH (the dotted lines in chart A) did not become perceptible till one hour, and then dropped to the same level, 7.2, usually before the end of the second hour, but sometimes later, and again remained stationary at this level. The solution of intermediate concentration was also intermediate in the curve of the pH change, this is not reproduced in the chart. Lowering of the temperature greatly slows the change, for at 25°C. this had not started at the end of three hours, even with the concentrated solution; but by the next morning all the solutions, including the most dilute, had reached the 7.2 level, at which they remained. In any case, the pH does not fall below 7.2; and if the pH is adjusted at or below 7.2, by the addition of hydrochloric acid, immediately after the phosphate is added, there is no change in either direction, nor any turbidity. This has an important application in the preparation of Fleisch's solution, in which the phosphate is added as phosphoric acid, which is then neutralized with sodium carbonate or hydroxide. In this case it is immaterial how rapidly and in what concentration the phosphoric acid is added, since no reaction occurs in the acid solution; and there is less chance of starting precipitation by the alkali, since this is immediately neutralized by the acid. However, with rapid addition of the alkali, the change may start within two or three hours; and in any case the mixtures deteriorate eventually, especially if in contact with ordinary glass, which supplies local foci of alkali. If carbonate, or better bicarbonate, is used for neutralization, the change is delayed, as will be presently explained.

The following charts compare the pH changes in the test tube at 38°C. (solid lines), with those in the aerated Magnus bath with contracting intestinal segment (broken lines), the phosphate being added in concentrated solution, i.e., dissolved in 0.1 cc. of water. In chart B the final concentration of sodium phosphate was 0.024 per cent. In the test tube the change occurs between two and three hours, i.e., quite late; in the presence of the intestines, there was an immediate fall, but this was probably due to the presence of free acid in the intestines.

In the remaining charts, the final concentration of sodium phosphate was 0.048 per cent. Chart C shows that the change occurs between $\frac{1}{2}$ and 2 hours, much more promptly, and also more extensively, than with 0.024

per cent. In chart D, 0.008 per cent of sodium bicarbonate was added before the phosphate: this delays the pH fall very materially, to between two and three hours in the test-tube. In the Magnus arrangement the fall appears quite prevented, presumably because when NaH_2PO_4 is formed, it liberates CO_2, which is removed by the aeration.

Difference of contraction type in fresh and old solutions. When the segments are placed in Locke solution to which sodium phosphate (0.048 per cent) has just been added, the contractions are at first pendular and change only gradually to the periodic type, as was illustrated in figure 1. It may be seen that this change begins before there has been a notable fall of pH, but that the functional change increases progressively with the pH fall. This suggests that both changes are due to the ageing of the solution. This was confirmed directly by placing a segment alternately in Locke solution immediately after the addition of the phosphate and in solution that had been mixed with phosphate several hours or days in advance; in the latter case the solution was generally filtered before it was used. Figure 3 A shows that on transferring from the fresh solution to one that had been aged for two days, the type changes immediately from the pendular to the periodic. This is independent of the difference of pH, for the same difference exists if the pH of the aged solution had been adjusted to that of the fresh solution by the addition of NaOH (fig. 3 B); and if the NaOH is added while the segment is contracting in the aged solution it does not alter the periodic type of contraction in the least. The time for which a solution must be aged to produce this immediate change to periodic contractions depends, as does the pH change, on the manner in which the phosphate is added to the Locke solution. If it is added as 0.1 cc. of a 24 per cent solution per 50 cc., ageing for two hours suffices (fig. 2 B).

Relation between the functional effects and pH changes. Although the development of periodic contractions goes hand in hand with the fall of pH, it is clear that the pH is not their cause; for they occur equally well if the pH has been restored, as in figure 3 B; they may start before the pH has fallen notably, as in figure 1; and in the absence of phosphate, decrease of pH from 8.2 to 6.8 does not provoke periodic contractions. The connection between the fall of pH and the development of periodicity is therefore casual, not causal; both are the effects of the phosphate, but are otherwise independent of each other.

Effects of increasing quantities of phosphate. Increase of the percentage of phosphate hastens the changes that lead to the fall of pH and the periodic functionation; and if the solutions are aged, the periodicity changes somewhat in type, according to the amount of phosphate that had been added. However, if the solutions are tested within half an hour after mixing, transferring the segments from one solution to the other (restoring the pH to 7.6 after each addition), periodicity does not appear even in

0.096 per cent phosphate. Under these conditions, 0.024 per cent to 0.072 per cent produces merely some increase of diastolic tone, and 0.096 per cent may be somewhat depressant (similar to fig. 4). From this, it appears clearly that the change to periodicity is not a direct effect of the phosphate as such. Since it has already been shown that it is not due to fall of pH as such, the cause must be sought in another direction, namely, in the removal of calcium. Before turning to this problem, however, it appears desirable to study the antagonism of bicarbonate to the phosphate.

Antagonism of bicarbonate and phosphate. The characteristic effects of the phosphate, namely, the periodic contractions and the fall of pH are both completely prevented or removed by the presence of sodium bicarbonate; 0.008 per cent of bicarbonate suffices to neutralize the usual concentrations of phosphate. With segments contracting in a Locke solution containing 0.008 per cent of sodium bicarbonate, the addition of phosphate is merely more or less stimulant, producing some increase of tone and to a less degree of rate, while the amplitude is either also increased, or decreases reciprocally to the rate or tone; but there is no tendency to periodicity even after several hours. The stimulation is generally well marked with 0.024 per cent of the phosphate, reaches its maximum about 0.048 per cent, then either remains constant to 0.096 per cent or decreases slightly (fig. 4). This corresponds quite closely to the early effects of phosphate in fresh solutions without bicarbonate; but as has been said, in the presence of the bicarbonate, 0.048 per cent of phosphate fails to produce periodicity even after several hours and the pH at the end of two hours remained at 7.8, while in the absence of bicarbonate it would have fallen to 7.5.

Conversely, if the periodic contractions have been established by phosphate solutions, the addition of bicarbonate converts these promptly into the pendular type. Figure 5, A and B, illustrates the difference according to whether the pH of such solutions is restored by bicarbonate (A) or NaOH (B). Again, 0.008 per cent of the bicarbonate appears sufficient to prevent or remove the periodic functionation resulting from 0.048 phosphate; and increasing the $NaHCO_3$ progressively from 0.008 to 0.042 per cent merely intensifies the bicarbonate effects, just as do similar increases of bicarbonate in phosphate-free Locke solution adjusted to the pH of 8.2; namely, progressive increase of the pendular rate (the most characteristic effect of bicarbonate) and decrease of amplitude and tone (mainly as the result of the rather excessive alkalinity). The antagonistic effect of the bicarbonate to the functional effect of phosphate is paralleled by a similar antagonism to the fall of pH (fig. 2 D). From this it may be concluded that the bicarbonate action is partly a chemical mechanism, preventing the precipitation of the calcium and possibly tending to redissolve the precipitate. The latter, however, is rather improbable; and since the bicarbonate may be effective also in solutions that have been

filtered after ageing (fig. 5 B), its action must be partly functional, i.e., the bicarbonate stimulation tends to convert periodic into pendular contractions. However, this can only occur if the decalcification has not progressed too far; for it will be shown later that the addition of bicarbonate to calcium-free Locke solution merely intensifies the periodic contractions without changing their type. The presence of bicarbonate also explains the absence or delay of periodicity in the customary saline solutions that contain phosphate, such as the Tyrode and the Fleisch solutions; and it contributes doubtless to the stability of the calcium and phosphate ions in the blood.

Glycocoll does not have this protective effect. When segments are placed in Locke solution containing 0.075 per cent of glycocoll, either at its natural pH of 7.2, or after alkalization to 7.8 by NaOH, the contractions are generally pendular. When 0.048 per cent of sodium phosphate is added, as 24 per cent solution, there is first the increase of tone and rate which is characteristic of phosphate as such; the contractions then become gradually periodic; and the pH falls to 7.2, if it had been previously raised by NaOH.

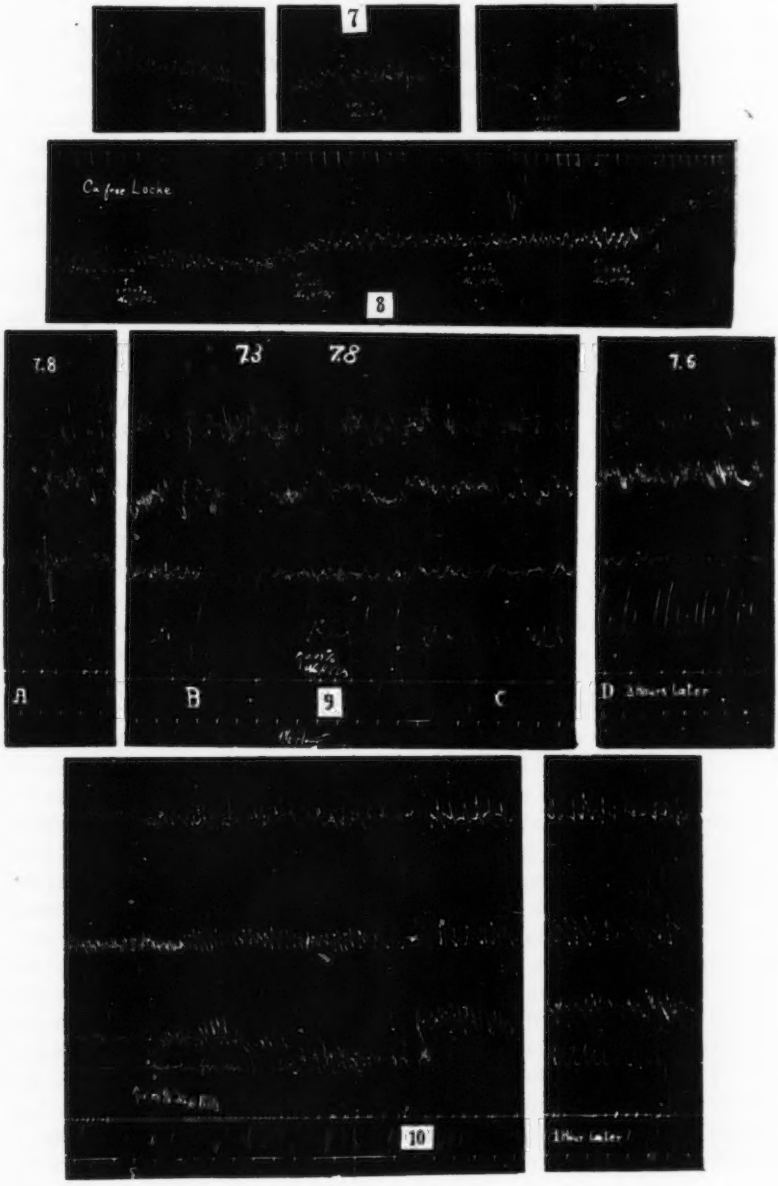
Decalcificaton as the cause of the phosphate periodicity. It may be advisable to recapitulate the subject to this point: The addition of sodium phosphate to Locke solution in which Magnus segments of rabbit's small intestines are suspended tends to change the type of contractions from pendular to periodic. This change is dependent on the ageing of the solution, and is hastened by increase of the concentration of phosphate; by adding it in concentrated solution; and by alkalinity, local as well as general. It is delayed by bicarbonate. The change is paralleled by fall of pH, but this is not a causative factor. Nor does the phosphate as such produce the effect; for fresh phosphate solutions produce merely increase of tone and rate of the pendular contractions, without any periodicity. Since the effect is due neither to the pH nor to the phosphate as such, there remains of the possible causative factors the decrease of calcium ions, as suggested by the precipitation. The phenomena were therefore compared with those that occur in Locke solutions containing deficient quantities of calcium. This has been made the subject of special in-

Fig. 7. Periodic contractions in calcium-free Locke solution are stimulated, but not changed in type, by the addition of increasing quantities of bicarbonate.

Fig. 8. Effects of increasing amounts of sodium phosphate on the periodic contractions in calcium-free Locke solution.

Fig. 9. Long, perfused intestines in fresh phosphated (0.048 per cent) Locke solution; the time tracing in this and the next figure is in minutes. The time since the start of the perfusion is noted on the tracing. Sodium bicarbonate, 0.03 per cent is added between B and C.

Fig. 10. Long perfused intestines in aged and filtered phosphate (0.048 per cent) Locke solution; 0.03 per cent of bicarbonate is added at the arrow.


7
Ca free Locke
8
7.8
7.3
7.8
7.6
A
B
9
C
D 3 hours later
10
1 hour later

vestigations, which will be published by R. Whitehead. It may suffice to state at present that decrease of the percentage of calcium to a fourth of the ordinary quantity (namely, to 0.006 per cent of $CaCl_2 \cdot 2H_2O$) produces merely some decrease of the rate and tone, and increase of the amplitude, without changing the pendular type; but if the calcium content falls materially lower, the contractions become periodic, exactly as with the addition of phosphate. The tone and the height of the contractions are also materially lowered. Restoration of the calcium to a fourth or more of the normal promptly changes the periodic back to the pendular rhythm.

Addition of bicarbonate and phosphate to the calcium-free segments. The calcium-free Locke solution also offers the opportunity of studying the pure actions of bicarbonate and of phosphate, uncomplicated by any effects that they might have on the state of the calcium in the ordinary Locke solution. Figure 7 shows the periodic contractions in calcium-free Locke solution adjusted by NaOH to pH 8.2; and the response to successive additions of bicarbonate. It will be seen that the bicarbonate does not remove the periodicity, but increases the amplitude of the periodic movements and tends to add longer tone waves, and generally also raises the tone. In other words, bicarbonate tends to stimulate whatever movements are present, the periodic in the absence of calcium, the pendular in the presence of calcium.

The addition of phosphate to the calcium-free solution, adjusted to pH 7.6 by the NaOH, also stimulates the periodic contractions and especially the tone, increasing with the percentage of the phosphate and more markedly than does bicarbonate. The type remains periodic (fig. 8). If the calcium-free Locke solution already contains bicarbonate (0.008 per cent), the addition of the phosphate (0.024 and 0.048 per cent) produces further increase of the amplitude and especially of the tone, i.e., the bicarbonate and phosphate stimulation are additive in the same direction.

The addition of calcium to phosphated Locke solution. The final test that the periodicity is due to decalcification could be furnished by replacing the precipitated calcium, by the addition of calcium chloride to the phosphated Locke solution. The success is illustrated in figure 6; at the left, the segment in Locke solution containing 0.048 per cent of sodium phosphate shows the characteristic periodicity, which is changed immediately to the pendular type when 0.071 per cent of $CaCl_2 \cdot 2H_2O$ is added. When the calcium chloride was added in six successive fractions, each of 0.008 per cent, i.e., a third of the "normal" concentration of 0.024 per cent, the transformation to the pendular rhythm started with the first dose, but progressed further with each additional dose. The calcium also produced progressive increase of rate and tone, especially systolic. The excursions increased with the first and second doses, then generally declined, reciprocal

to the rate. The first dose of calcium also lowered the pH from 7.8 to 7.2, at which level it remained with the further additions. If the pH of the phosphated Locke solution had been adjusted at 7.3 by the addition of the phosphoric acid, the addition of 0.071 per cent of the calcium chloride lowered it at once to 6.3 or 6.5. In other words, the addition of the calcium chloride lowers the pH of phosphated Locke solution by about 0.6 to 1.0 point. Since the addition of calcium chloride to plain or bicarbonated Locke solution does not lower the pH, it is evident that the calcium reacts with the phosphate, presumably with the formation of $Ca_3(PO_4)_2$ and NaH_2PO_4. The acidity that develops is not responsible for the functional change, as has been shown previously, and accordingly realkalinization to 7.8 by the addition of NaOH or bicarbonate does not restore the periodic type. On the contrary, the addition of bicarbonate to the phosphate mixture before adding the calcium seemed to produce sensitization of the intestines to the spastic action to the full doses of calcium, sometimes with fibrillary contractions. This is probably an intensification of the calcium action, due to the liberation of the calcium that had been bound by the phosphate.

Effect of phosphate and decalcification on the peristaltic efficiency. This was tested on the long excised intestines, with lumen perfusion, arranged as described by von Oettingen, Sollmann and Ishikawa, 1928. Figure 9 (A) shows that the peristaltic contractions are quite normal in freshly phosphated (0.048 per cent in diluted form) Locke solution, but that they soon deteriorate progressively, so that peristalsis and propulsion are greatly slowed (B). Addition of sodium bicarbonate, 0.03 per cent to the bath, produces immediate improvement (C), which is maintained to the end of the experiment, about three hours (D). In figure 10, the intestines were placed in a phosphated (0.048 per cent) Locke solution that had been aged for two days and filtered. This did not permit peristalsis, so that the contractions, at the left of the tracing, are purely pendular; but the addition of 0.03 per cent sodium bicarbonate initiates vigorous peristalsis which lasted till the experiment was terminated in about an hour.

These experiments show clearly that phosphate decalcification interferes with peristalsis even more than with the pendular movements; and confirm that the periodic contractions in the Magnus segments are not peristaltic, but consist of superimposed incomplete pendular contractions. In other words, the effects of decalcification on intestinal movements are purely depressant.

SUMMARY AND CONCLUSIONS

The immediate effect of the addition of small quantities of sodium phosphate to Locke solution tends to improve the movements of the excised intestines; although not as much as does sodium bicarbonate. Concentrations above 0.048 are directly depressant.

Sooner or later, however, and often in the course of the experiment, the phosphate solutions, the dilute as well as the concentrated, undergo chemical changes, manifested by fall of pH and the development of turbidity or precipitation. This is accompanied by depression of the contractions, which in the Magnus segments leads to a characteristic type of periodic contractions, formed by the superposition of incomplete pendular contractions. In lumen-perfused intestines, the depression involves especially the peristaltic activity, which may be completely abolished when the pendular contractions are still fairly active.

The development of this alteration in the solution is hastened by heat; by concentration of the phosphate, localized as well as general, and by alkalinity. It is delayed by the presence of bicarbonate.

The functional change is not due to the alteration of the pH, nor to the phosphate as such, but to the precipitation of calcium. The phenomena are entirely analogous to those of extensive decalcification, and they can be removed by the addition of calcium to the solution.

Sodium bicarbonate delays the chemical changes, and also counteracts the functional depression by stimulation; provided that some calcium is present.

The addition of phosphate to Locke or Ringer solution therefore tends to reduce the calcium concentration to a varying and unpredictable degree. This danger may be minimized by using only freshly prepared mixtures, and by adding the minimum of phosphate (preferably not more than 0.024 per cent) in as dilute a form as possible, best as phosphoric acid or acid phosphate with subsequent neutralization with sodium bicarbonate solution; but there remains generally some risk of uncontrollable alteration if the experiment is prolonged.

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THE DURATION OF VENTRICULAR RESPONSE, MECHANICALLY AND ELECTRICALLY RECORDED, AS INFLUENCED BY RATE, INITIAL TENSION AND FATIGUE

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The effect of varying rates and volumes upon the duration of the phases of the isometric contractions of the ventricle has frequently been investigated. The influence of these factors upon the duration of the electrogram and particularly the correlation of mechanical and electrical changes have not been so fully determined. It is with this correlation, and with certain effects of fatigue that this paper deals.

When the heart rate is increased in the frog, the turtle, or the mammal, the different observers are in essential agreement that both systole and diastole are shortened, the latter more than the former (Hürthle, 1891; Porter, 1892; MacWilliam, 1888; Hunt, 1899; Lombard and Cope, 1917, 1919, 1926; Wiggers and Clough, 1919, and others). Equally consistent are the findings which show a similar relationship between cycle lengths and the duration of ventricular negativity (Samojloff, 1910; Bazett, 1918; Mines, 1913, and others).

In the cold-blooded heart it has generally been reported that an increase in the diastolic volume of the heart increases the duration of systole, of relaxation, or of both phases as a whole (Frank, 1895; Kozawa, 1915; Daly, 1923; Segall and Anrep, 1926 and others). This effect is observed for both isotonic and isometric contractions. For the mammalian heart, however, the results of different investigators have not been so consistent. Patterson, Piper and Starling (1914), using the dog heart-lung preparation, reported an increase in the duration of systole with augmented venous return or with raised arterial volume. Katz (1921), with similar procedures on the entire animal, obtained comparable results. The heart rates in these investigations were, of course, carefully controlled. Bowen (1904) observed an immediate increase in the duration of systole in man after the onset of, and a sudden shortening at the end of exercise. He believes these changes are attributable to changes in venous return and arterial resistance with consequent alterations in ventricular volume. Bartos and Burstein (1924) report a lengthening of mechanical systole with intravenous infusion of saline solution.

In contrast with these results, Wiggers and Katz (1922) and Bartos and Burstein (1924) report that compression of the abdominal or thoracic aorta in the dog, a procedure which increases the diastolic volume of the ventricle without raising venous pressure, shortens the systole.

In the cold-blooded heart, the relation between the electrical and mechanical durations of systole, as affected by changes in volume, has been studied by Daly (1923). With augmented volumes, in two experiments, Daly reports increases in the durations of the phase of rising tension of 7.3 and nearly 27 per cent respectively. The corresponding prolongations in the durations of the electrograms were about 62 (monophasic) and 79 (diphasic) per cent. The durations of mechanical response (from the beginning of contraction to half relaxation) increased 25 and 36 per cent. The increase in the lengths of the electrical response is surprisingly great.

Bartos and Burstein (1924), working on the dog, studied the relation between the durations of systole as measured from heart sound records, and the duration of the ventricular complex of the electrocardiogram. With augmented aortic resistance they state that "the outstanding feature was the constancy of the electrical systole while the mechanical systole shortens." Katz and Weinman (1927) report that "increasing the arterial resistance caused a decrease in the duration of ejection and of the Q-T interval but the amount of shortening was not equivalent in most of the experiments of this group; the effect on mechanical was greater so that after compression the T wave occurred later in relation to the ends of ejection. . . . . In a few instances the effect was just the reverse."

The work we report includes experiments upon isolated, perfused turtle ventricles and upon turtle and dog hearts *in situ*.

METHOD FOR TURTLE EXPERIMENTS. The first series of experiments was performed on the ventricles of eighteen turtles. Each ventricle was excised and arrangements made to record electrograms and either isotonic or isometric contractions. The method of recording the contractions was similar to that of Kozawa (1915). Strongly buffered, well-oxygenated Ringer's solution (pH 7.5 to 7.8) was siphoned, as needed, into a burette. The burette was connected, by way of a stopcock, with a small tambour, over which a rubber membrane was tightly stretched. The tambour in turn was connected by heavy rubber tubing with a cannula which was inserted into the ventricle through the A-V valve. A waxed thread at the A-V groove, as near the ventricle as possible, held this cannula in place. A smaller cannula, inserted into an aorta, and connected by heavy rubber tubing with a stopcock, served to permit emptying of the ventricle. For isometric contractions, the outlet stopcock was closed and any desired volume of solution was run into the ventricle from the burette. The inlet stopcock was then turned and the ventricle contracted against a closed system. The movement of the tambour membrane was recorded by a light

right-angled lever in front of the camera slit. For isotonic contractions both stopcocks were opened and the solution allowed to run in slowly from the burette.

The ventricle was partly immersed in a glass dish containing Ringer's solution of the same composition as that used for perfusion. Strands of absorbent cotton, one upon the ventricle and one dipping into the solution in the dish, led to nonpolarizable electrodes of the "boot" type containing zinc sulfate and amalgamated zinc strips. Thence the action current was led to the string galvanometer. The ventricle was stimulated near the base at any desired rate by break shocks applied by way of closely adjoining needle electrodes. By this arrangement, the mechanical and electrical responses could be simultaneously photographed.

Before recording, the ventricle was perfused for several minutes. The inlet stopcock was closed and the ventricle allowed to empty. The outlet stopcock was then closed and, without loss of time, some ten to twenty responses were recorded at varying cycle lengths. A measured volume of perfusate was immediately admitted, the system closed, and another series of responses recorded. Continuing in this manner, after two, three or four additional volume increments, the ventricle was allowed to empty and a series obtained to serve as a control. In some cases, the sequence of filling was reversed in that the first filling was the greatest, and, after each ten or twenty contractions, the ventricle was allowed to eject a measured volume of perfusate.

As a check, a series of experiments was performed on turtle hearts *in situ*. The pericardium was opened, the frenum cut and attached to the lever before the camera slit. Nonpolarizable electrodes were placed, one on the ventricle, one on the body wall. No attempt was made to vary rate, all records being taken while the heart was beating regularly in response to sinus impulses. The amount of blood in the ventricle was varied by clamping the arteries leading from the heart.

RESULTS OF THE TURTLE EXPERIMENTS. The results of the turtle heart experiments enable us to make certain definite statements in regard to the influence of the mechanical and of the "chemical" condition of the muscle upon the phases of rising and falling tension, the tension developed during contraction, and the duration of the electrogram. By the mechanical condition we mean the fiber length as determined by the degree of filling or initial stretch; by "chemical" condition, we designate, for want of a better term, the influence of rate, fatigue, etc. When a systole begins before the disappearance of the tension produced during a previous contraction, we are probably dealing with a special case of a change in chemical condition. The relative refractory period has not yet disappeared.

The influence of augmented ventricular volume, under approximately isometric conditions, is a definite prolongation of the phase of rising ten-

sion, together with a more marked extension of the phase of falling tension. In figure 1, the results of a typical experiment are shown. The average increase in the duration of the phase of rising tension is 40.9 per cent; of mechanical change to half relaxation 101.5 per cent; of the total

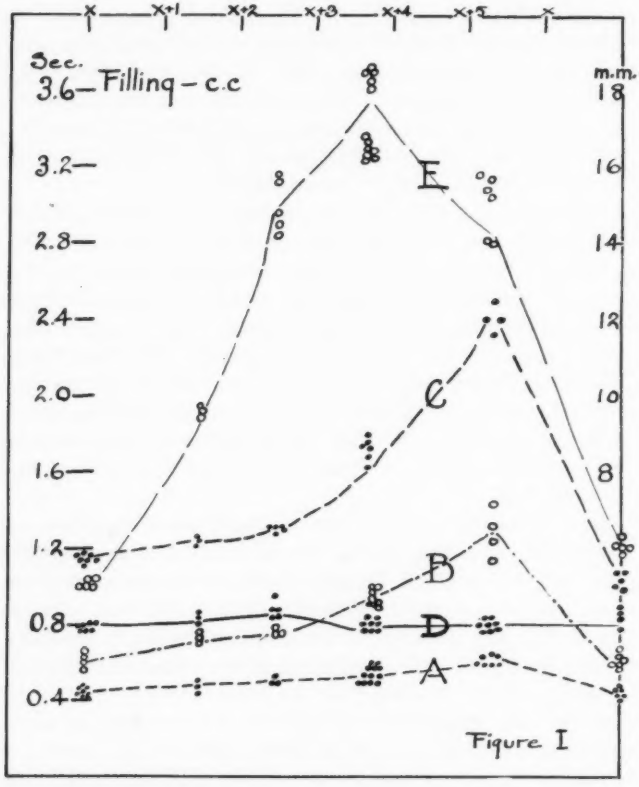


Fig. 1. Abscissae represent filling, x being the volume remaining in the heart which had emptied itself. The points on the extreme right represent durations obtained at end of experiment when heart was again empty. Ordinates represent the durations, the scattering of points resulting from inclusion of somewhat variable cycle lengths. *A*, phase of rising tension; *B*, mechanical change to half-relaxation; *C*, total mechanical change; *D*, total electrical; *E*, amplitude in millimeters.

mechanical change 106.8 per cent. Another figure from a different experiment will be found in our preliminary report (Hafkesbring and Ashman, 1927). As others have noted, the phase of falling tension is much more prolonged than that of rising tension.

In contrast with these definite increases in the mechanical responses, the duration of the electrogram remains either unchanged, or the changes are within the limits of experimental error (fig. 1, curve *D*). This result was somewhat unexpected in view of the previous work of Daly (1923). The only argument which we can advance against the acceptance of our results is the decrease in amplitude of the electrograms which resulted from increased fillings. It thus became difficult to determine the end point of the electrogram at the larger fillings; but it is very unlikely that

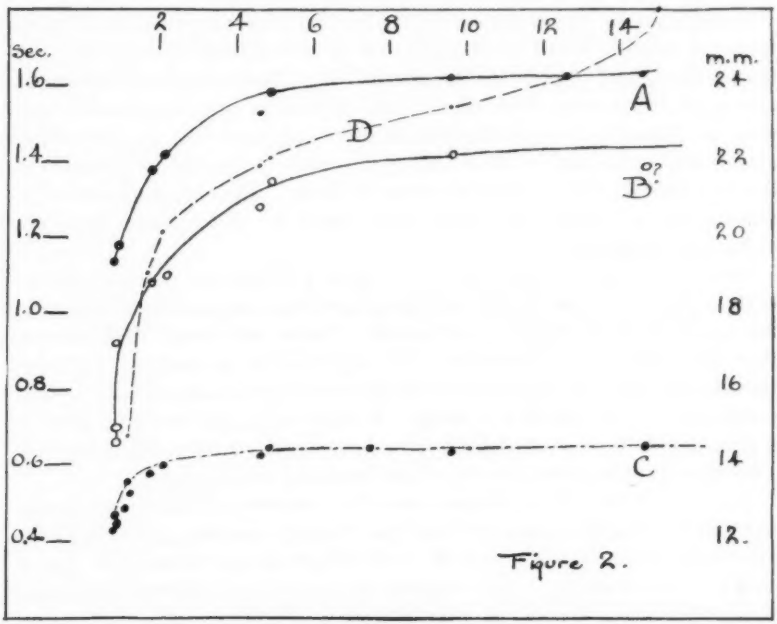


Fig. 2. Abscissae, cycle lengths; ordinates, durations of the phase; *A*, total mechanical response; *B*, electrogram (R to end of T); *C*, phase of rising tension; *D*, amplitude in millimeters.

any change approaching the mechanical in magnitude would have remained undetected.

Had there been a progressive diminution in the duration of QRS with augmented fillings, the total electrogram might, for that reason, have remained unchanged; but as a matter of fact, QRS remained remarkably constant. Moreover, our measurements of the duration of the total electrical change are checked against the S-Te (apex of A to end of T) and the S-Ta (apex of S to the apex of T) intervals. The S-Ta measurements

were taken only in those cases showing no change in the form of the T wave.

Our experiments upon the turtle heart *in situ*, experiments in which excellent electrograms were obtained, completely confirm our perfusion experiments. Here, however, the contractions were isotonic before and approached the isometric after the volume increase was induced. As in the isolated heart, the mechanical responses increase in duration when the ventricular volume is augmented by clamping the arteries leading from the heart; the electrical changes are of constant duration.

Because of the completeness of previous work on the subject, we have been less concerned with the influence of cycle length upon the duration of the electrical and mechanical responses. It is, however, worth noting at this point, that since these changes are typical in our experiments, and since no difficulty was encountered in detecting them (fig. 2), they serve as a check upon and confirm the trustworthiness of our measurements directed toward determining the effects of filling. The changes induced by varying the cycle length are illustrated in figure 2. No written exposition of them is necessary.

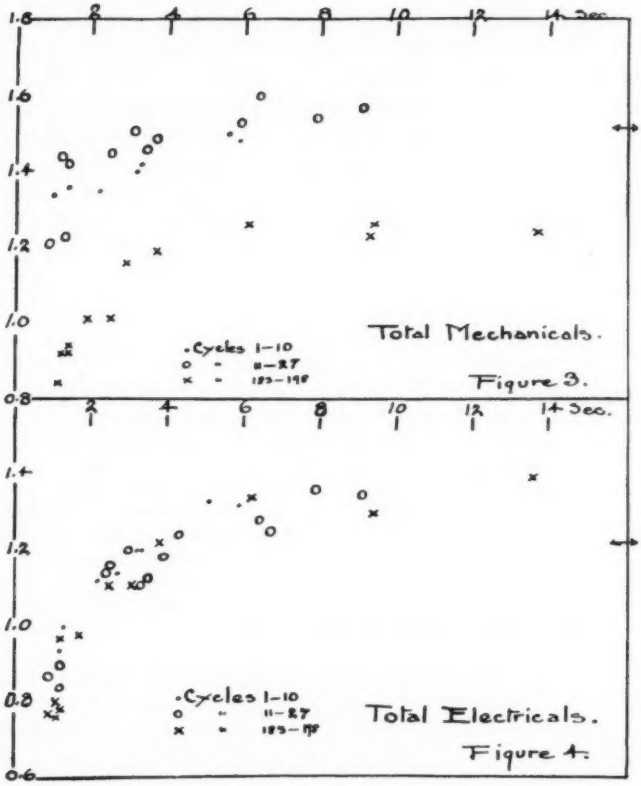
Curve *D* of figure 2 and curve *E* of figure 1 are graphic representations of the influence of cycle length and filling upon the tension developed during the approximately isometric contraction. Segall and Anrep (1926) report that the development of tension in the frog ventricle is maximal, provided the response does not begin before the previous response is complete. Our results tend to confirm their findings. If there is fatigue, however, there is a progressive increase in the development of tension even when the new contraction begins after the end of the foregoing relaxation.

A moderate cumulative fatigue, caused by responses of such frequency as to preclude complete recovery, has been found to produce a very striking diminution in duration of systole, both electrical and mechanical (figs. 3 and 4). For example, if the ventricle has rested or has been responding at a low rate for a variable length of time, e.g., a minute, and is then stimulated several times at, say, 5 second intervals, the electrical and mechanical systoles will be distinctly longer than if the stimuli follow a series of responses at a rapid rate. This effect is sometimes practically absent in the well-perfused ventricle; it is, in our experience, invariably present when the perfusion is inadequate. Gilson[1] has pointed out the analogy between the progressive shortening of electrical systole, thus produced, and the treppe phenomenon. Our data do not permit us to decide whether the phenomenon is strictly comparable to the treppe; they demonstate only that, like the treppe, it is a result of activity under conditions which will cause fatigue.

Our results do not make possible a quantitative comparison under iso-

[1] Personal communication.

tonic and isometric conditions of the phenomena we have studied. Qualitatively the effects are the same under the two conditions. It therefore appears unnecessary to increase the length of this paper by a separate discussion of the isotonic responses. Our experiments on the dog heart, described below, were all carried out under isotonic conditions. Their



Figs. 3 and 4. Abscissae, cycle lengths; ordinates, durations of the phase

qualitative agreement, so far as they go, with the isometric responses of the turtle heart will be apparent.

METHOD FOR DOG EXPERIMENTS. Two different procedures were followed. In the first, the animals were prepared for the operation, using ether anesthesia, and tracheotomized for artificial respiration. The heart was exposed by opening the thorax and pericardium. A cannula was connected by heavy rubber tubing to a very small tambour over

which a heavy, tight rubber membrane was stretched. A tiny mirror on the surface of the membrane reflected a beam of light from a lantern to the camera slit, so that movements of the membrane caused by variations of pressure in the artery could be photographed simultaneously with the record of the action current. Electrocardiograms were obtained instead of electrograms. Small metal discs with binding posts were attached just under the skin of the right foreleg and left hind-leg (lead II) and were connected to the electrocardiograph. The arterial resistance was raised by compression of the arch of the aorta or of the thoracic aorta.

Records were obtained before, during and after compression. Each procedure was repeated several times. The vagi were usually cut.

With the second procedure, a larger series of experiments was performed, using heart sounds instead of pressure changes as the criterion of the duration of ventricular systole. The method was essentially similar to that

TABLE 1

Dog 3. February 24, 1923

CYCLE LENGTHS R-R	ELECTRICAL		MECHANICAL Q-Sys-e	REMARKS
	Q-Ter	S-Te		
0.381	0.218	0.180	0.216	No compression
0.382	0.212	0.173	0.227	Compression of the arch
0.382	0.213	0.183	0.213	No compression
0.386	0.210	0.176	0.233	Compression; arch
0.387	0.212	0.176	0.214	Immediately after release
0.385	0.218	0.185	0.213	Approximately one minute later
0.390	0.218	0.186	0.236	Compression; arch
0.385	0.221	0.188	0.212	Compression released

described by Wiggers and Dean (1917). A heart sound record and electrocardiogram (lead from right shoulder to left side at about the level of the eighth or ninth rib) were simultaneouly obtained before, during and after compression of the abdominal aorta or infusion of warm Ringer's solution into the right jugular vein. The quantity of Ringer's used varied from 50 to 90 cc. introduced within 8.5 to 16 seconds. The animals were anesthetized with morphine (0.5 cc. per kilogram of a 2 per cent solution) and chloretone (0.5 cc. per kilogram of a 10 per cent solution in ether and petrolatum). The thorax not being open, artificial respiration was not employed. The vagi were left intact for one record by each procedure, then cut.

RESULTS OF DOG EXPERIMENTS. The results of the dog experiments have been, on the whole, less striking than with the turtle, but have led to one main conclusion in agreement with our finding on the turtle: changes

in ventricular volume, even when they alter the duration of systole do not produce parallel changes in the duration of the electrocardiogram. We have, as already mentioned, employed two methods of augmenting the volume: compression of the thoracic or abdominal aorta, and rapid infusion of warm saline solution into an external jugular. Our results are almost equally divided between those in which there was an increase in mechanical systole and those in which no appreciable change occurred. In no case, in which there was prolongation of systole, did the electrocardiogram (either Q-Te or S-Te) increase by more than the probable error in our measurements, although, in some cases, the electrical response decreased. Possible reasons for the decrease will be given below.

These results are illustrated in tables 1, 2 and 3.

The two tables (1 and 2) show that the heart rate (cycle length) in each case is approximately the same throughout the experiment. As illustrated in these tables, with constant cycle lengths, the duration of the

TABLE 2

Dog 4. March 8, 1928

CYCLE LENGTHS R-R	ELECTRICAL		MECHANICAL R-Sys e	REMARKS
	R-Ter	R-Te		
0.310	0.142	0.198	0.248	No compression
0.312	0.142	0.194	0.272	Compression of thoracic aorta
0.314	0.144	0.199	0.242	No compression
0.309	0.141	0.199	0.272	Compression on
0.307	0.142	0.198	0.238	No compression

mechanical response increases very slightly, but definitely, with compression of the arch of the aorta, or of the thoracic aorta. For the experiments on dog 3, with compression of arch of the aorta, the increase in the duration of the mechanical response was from 0.216 to 0.236 second, or 9.2 per cent. For dog 4, which shows the results of compression of the thoracic aorta, the increase was from 0.242 to 0.272 second, or 12.4 per cent.

In contrast to this increase in the duration of the mechanical responses of 9.2 and 12.4 per cent, respectively, the duration of the electrical responses under the same conditions shows very slight changes, if any at all. In experiments on dog 3, the durations of the electrical responses range from 0.213 to 0.218 second with no compression, to 0.210 to 0.218 second with compression. If these slight changes can be interpreted in any other way than as a slight error in measurement, then there is a slight decrease, rather than an increase, in the duration of the electrical response. The results of the experiments for dog 4, table 2, are similar. The duration of the electricals varied from 0.142 to 0.141 second with compression, then back to 0.144 second when the compression of the thoracic aorta was released.

TABLE 3

Dog 16. Weight 9 kgm.

EXPERIMENT NUMBER	CYCLE	Q-Te	S-Te	HEART SOUNDS	REMARKS
					Vagi intact
1	0.588	0.235	0.204	0.170	Normal
	0.622	0.235	0.202	0.171	Normal
	0.607	0.236	0.204	0.179	Infusion started 90 cc. 38.75°C.
	0.619	0.231	0.202	0.179	Infusion continued
	0.577	0.229	0.200	0.183	Infusion continued
	0.447	0.212	0.184	0.177	Infusion continued
	0.427	0.210	0.181	0.172	Infusion continued (ending)
	0.396	0.206	0.179	0.160	Normal
	0.427	0.206	0.177	0.164	Normal
2	0.419	0.205	0.176	0.163	Normal
	0.459	0.205	0.177	0.162	Normal
	0.518	0.209	0.179	0.170	Compression begun
	0.664	0.216	0.181	0.185	Compression continued
	0.523	0.210	0.181	0.185	Immediately after release
	0.475	0.210	0.181	0.166	Normal
					Vagi cut
3	0.294	0.174	0.147	0.133	Normal
	0.294	0.178	0.149	0.129	Normal
	0.295	0.173	0.145	0.134	Infusion started 90 cc. 38°C.
	0.300	0.173	0.141	0.143	Infusion continued
	0.295	0.167	0.139	0.147	Infusion continued
	0.292	0.167	0.138	0.149	Infusion continued
	0.292	0.162	0.136	0.147	Infusion stopping
	0.287	0.163	0.137	0.143	20 sec. later
4	0.289	0.160	0.134	0.143	Normal
	0.284	0.161	0.132	0.147	Normal
	0.281	0.158	0.128	0.158	Compression
	0.286	0.160	0.131	0.141	Compression continued
	0.289	0.158	0.129	0.144	Compression continued
	0.290	0.157	0.129	0.154	Compression continued
	0.294	0.163	0.135	0.143	Normal
	0.289	0.162	0.133	0.149	Normal
	0.285	0.159	0.130	0.148	Normal
5	0.273	0.159	0.132	0.129	Normal
	0.278	0.158	0.130	0.128	Normal
	0.274	0.158	0.128	0.137	Infusion started 90 cc. 37.5°C.
	0.279	0.156	0.127	0.139	Infusion continued
	0.277	0.149	0.122	0.142	Infusion continued
	0.274	0.150	0.122	0.144	Infusion continued
	0.272	0.152	0.124	0.142	Infusion stopping
	0.268	0.150	0.123	0.139	Normal after 15–20 seconds

TABLE 3—*Concluded*

EXPERIMENT NUMBER	CYCLE	Q-Te	S-Te	HEART SOUNDS	REMARKS
					Vagi cut
6	0.267	0.150	0.120	0.143	Normal
	0.276	0.151	0.122	0.150	Compression
	0.276	0.151	0.121	0.151	Compression continued
	0.278	0.148	0.120	0.152	Compression continued
	0.278	0.150	0.121	0.143	Normal
	0.279	0.145	0.118	0.152	Compression begun
	0.279	0.149	0.123	0.140	Compression continued
	0.281	0.155	0.126	0.145	Immediately after release
	0.277	0.152	0.125	0.143	Normal

The results with increased arterial resistance, showing an increase of from 8 to 12 per cent in the duration of the mechanical response, were consistent throughout the first series on dogs. These results agree with those of Daly (1923) on the dog heart, and with results obtained by Katz and Weinman (1927), which show that mechanical systole is affected more than the electrical when experimental conditions are altered.

Table 3 gives the complete results of a series of typical experiments on one dog, and includes both compressions of the abdominal aorta and venous infusions. In this animal, one procedure of each kind was carried out before and two of each after cutting the vagi. Each set of figures is the average of five cycles.

As illustrated by experiments 2, 4 and 6 of table 3, the duration of the mechanical response (heart sounds) increases with compression of the abdominal aorta. In experiment 4, with cycle lengths approximately equal (0.289 and 0.281 second), the increase is from 0.143 to 0.158 second, or 10.5 per cent. The results of the experiments using compression of the abdominal aorta to increase arterial resistance caused increases in the duration of the mechanical response varying from 8 to 13 per cent.

In experiment 4 the duration of the electrical response, both Q-Te and S-Te, shows a very slight decrease, the duration of Q-Te varying from 0.160 to 0.158 second and S-Te, from 0.134 to 0.128 second. Reasons for the diminution are suggested in the discussion.

Experiments 1, 3 and 5 of table 3 give in detail the results of typical experiments with the venous infusion of warm Ringer's solution. In experiment 5, 90 cc. of solution at 38.0 degrees C. was introduced in 16 seconds. The cycle length remained practically unchanged throughout the experiment, shifting only from 0.273 to 0.277, then back again to 0.268 second. The duration of the mechanical response increased from a normal of 0.129 to 0.144 second, or 11.62 per cent. The duration of the electrical response, both Q-Te and S-Te, decreased progressively. This effect is mentioned in the discussion.

Throughout the whole series of infusions upon seven dogs, mechanical systole was either increased in duration or unaffected. Electrical systoles were decreased or unaffected.

The grand averages of the infusion experiments upon the seven dogs[2] are as follows, the figures for "normal" representing averages before infusion was started, and "infusion," those during the procedure. The average cycle length in the two cases was approximately equal (0.471 and

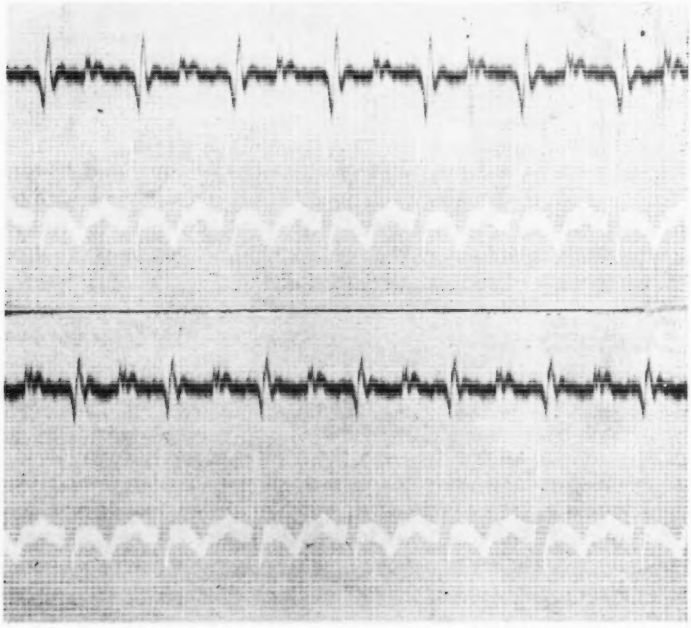


Fig. 5. Dog 16, tracing III. Simultaneous heart sound records and electrocardiograms taken before (upper curve) and during (lower curve) of infusion with warm Ringer's solution. Time in 0.1 and 0.02 sec.

0.466 second). The electrical response, as illustrated both by the Q-Te and S-Te intervals, remained practically unchanged, Q-Te varying from 0.2026 to 0.2022 second, and S-Te, from 0.1734 to 0.1724 second, a slight decrease. On the other hand, the duration of the mechanical response increased from 0.1810 to 0.1842 second. This is a very slight increase, but since these figures represent the grand average of all experiments performed

[2] Two other dogs yielded electrocardiograms or heart sound records which were not accurately measurable. They are therefore omitted.

with infusion, including experiments showing no change, we consider them significant.

So far as the influence of heart rate upon the duration of electrical and mechanical systole is concerned, our findings are in accord with those of previous investigators.

DISCUSSION. We have called attention to the striking effect of continuous activity upon the duration of the electrical and mechanical systole in the excised turtle heart. The activity to which we refer can be assumed to cause a slight degree of fatigue under the conditions of our experiments. The consequence of this fatigue is a diminution in the duration of systole, although cycle lengths remained unchanged. Clearly, therefore, a change in the chemical condition of the muscle has caused a modification of electrical and mechanical systole.

In both *turtle* and *mammalian* hearts, the contrast between the effect upon the duration of electrical and mechanical systole of rate or cycle length changes and volume changes may be explained by a simple assumption—that the chemical condition of the muscle, as modified by fatigue, by cycle length, or by the onset of the new response before the disappearance of the old one, influences the duration of both the electrical and mechanical responses; that the electrical is uninfluenced by further chemical changes occurring while the response is in *progress;* and that the mechanical is influenced not only by the chemical state of the muscle at the moment the response begins, but also by the changes which occur while the response is in progress. Thus an increased ventricular volume, which results in a greater liberation of energy and necessitates a greater oxgyen consumption for recovery (Starling and Visscher, 1926) will usually increase the duration of systole, but will leave the duration of the electrical systole unaffected.

If the average figures (mentioned above) for all our infusion experiments be taken, it will be observed that there is a slight decrease (0.57 per cent) in the S-Te interval with an increase (1.76 per cent) in the mechanical systole. This effect is much more striking in individual experiments than in the average. Reference to table 3 will show that the decrease is a progressive one. We suggest, as possible causes for the decrease, a progressive impairment of the condition of the heart resulting from the infusion, and in some cases, a slight increase in temperature.

Another phenomenon will also be observed in table 3. Generally, after infusion, the duration of mechanical systole remains increased to above the previous normal. This we interpret as a consequence of augmented blood volume, with persisting increased ventricular volume.

In regard to the development of electrical potential in the stretched muscle, we can arrive at no significant conclusion. The low amplitude of the electrogram in the well-filled turtle ventricle is probably merely a result of a short-circuiting of the action current through the greater volume

of fluid. In this connection, however, the findings of Fulton (1926) on skeletal muscle are of interest.

SUMMARY

1. In both turtle and dog ventricles, the duration of systole, electrical and mechanical, is increased by prolongation of the cardiac cycles.

2. The mechanical, but not the electrical systole, is increased by those procedures which increase the diastolic volume of the ventricles. This result, most clearly seen in the turtle heart, is independent of cycle length changes.

3. In the turtle heart, those conditions leading to cumulative fatigue shorten systole, both mechanical and electrical.

4. In the turtle heart, the duration of the phase of relaxation and the tensions developed in contraction are similarly augmented by increases in cycle length or in diastolic volume.

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EFFECT ON RESPIRATION, BLOOD PRESSURE, AND CAROTID PULSE OF VARIOUS INHALED AND INSUFFLATED VAPORS WHEN STIMULATING ONE CRANIAL NERVE AND VARIOUS COMBINATIONS OF CRANIAL NERVES

I. Branches of the Trigeminus Affected by These Stimulants

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The results to be reported in this investigation grew from some preliminary experiments on the peripheral and central pathways of smell in which Kratschmer's and Beyer's experiments on respiratory reflexes were repeated and expanded. At first my interest in this problem was concerned only with an attempt to find a volatile substance which would stimulate the olfactory nerve alone, so that in tracing out the pathways concerned in these reflexes, it would not be necessary to section the trigeminal and possibly other cranial nerves as a preliminary step in each experiment.

At the very outset we are confronted with the necessity and the simplest manner of producing an *olfactory animal*, by which is meant one in which the trigeminal nerves to the nasal region have been cut and the vagus and other nerves supplying the lower respiratory areas are not functioning. These experiments form the nucleus for the first report.

It is of vital importance in this problem not only to be able to isolate the reactions obtained from stimulation of the olfactory and trigeminal nerves, but to be certain that the reactions obtained from inhalation and insufflation of various vapors are not largely caused from humoral and mechanical stimulation of the lungs and blood vessels or to humoral stimulation of the medulla centers.

Direct effect of insufflation of xylol and ammonia vapor on the naso-ciliary nerve endings. This experiment was started by severance of both cerebral hemispheres directly behind the olfactory bulbs in rabbit (618), thus blocking all olfactory impulses. The right maxillary nerve was severed in the orbit. A day later the left maxillary nerve was sectioned. Pinching the nostril after the second operation demonstrated that the maxillary nerves were not functioning in these regions. Autopsy showed that all olfactory, maxillary and naso-ciliary fibers supplying the nasal mucosa

had been cut, the naso-ciliary nerves having been severed later in the experiment. The naso-ciliary nerves were probably the only general sensory nerves supplying the nasal mucosa during the first part of the experiment.

The day following the last operation the rabbit was given two-thirds of a full anesthesia dose of veronal-sodium intravenously. Under light ether anesthesia a mercury manometer was connected to the right carotid and a balloon respiratory recording apparatus was strapped to the thorax and abdomen. To eliminate the possibility of absorption of these vapors by the blood and the stimulation of the deeper respiratory organs, two tracheal cannulae were inserted into the trachea and pharynx respectively. The

Fig. 1. Thoracic respiratory and blood pressure tracings, insufflation into nostrils of a tracheotomized rabbit (row A) olfactory and maxillary fibers cut, space represents 22 seconds (row B) naso-ciliary nerves also sectioned.

lower one took care of ventilation and the upper one provided an outlet for the vapor blown into the nostrils and also prevented any vapor from entering the larynx. All exposed flesh was covered with a layer of cotton to eliminate stimulation from that source. A blowing bottle was used to force vapors into the nostrils so that they would come in contact with the olfactory epithelium. It was provided with a three-holed stopper and three glass tubes. One tube extended from a rubber vaporizing bulb to the bottom of the solution and the other two from the cork to the nostrils.

For control, air was blown through water into both nostrils for a period of considerable time. A graph not reproduced is identical to the control

graph of the next experiment (fig. 2 A). It discloses no change in respiration, blood pressure or carotid pulse.

The first test consisted in insufflating xylol into both nostrils, with the result that moderate changes take place in respiration, blood pressure, and carotid pulse (fig. 1 A). The respiratory excursions in the top tracing show some depression and inhibition, which continue for some time after insufflation ceased. The rise in blood pressure was 14 mm. The portion of the graph to the right of the space, an omitted portion of the record corresponding to 22 seconds, shows the blood pressure returning to normal. Before stimulation the carotid pulse rate was 8.5 beats in two seconds and during insufflation it dropped to 7.5 beats in two seconds. The height of

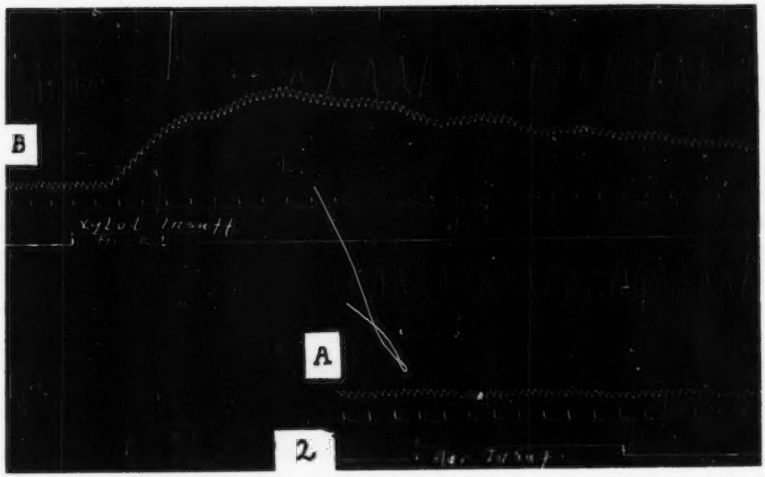

Fig. 2. Same as figure 1 except that naso-ciliary and olfactory fibers were sectioned and maxillary nerves were intact in both graphs.

the pulse waves before stimulation averaged 1 mm. and during stimulation they were 1.5 mm.

Another xylol record taken from this animal is practically a duplicate of the one shown.

In the second part of this experiment the naso-ciliary nerves were cut and the respiratory and blood pressure tracings (fig. 1 B) were taken during the insufflation of ammonia into both nostrils. It is obvious that there is no change in respiration, blood pressure or pulse in this graph. The insufflation of xylol into both nostrils also produced a graph in which there is no alteration of respiration or circulation.

The entire experiment was repeated in other animals with like results.

In rabbit (616) the projection of ammonia vapor into both nostrils before the naso-ciliary nerves were cut produced some depression and inhibition of respiration, a rise in blood pressure of 16 mm., an increase in the height of the pulse waves of 0.5 mm., a slowing of the rate of the pulse of one beat in two seconds, while absolutely no changes were effected in respiration or circulation after the naso-ciliary nerves were sectioned.

Direct effect from insufflation of xylol and ammonia vapors on the maxillary nerve endings. This experiment was conducted after exactly the same manner as the first experiment, excepting that both maxillary nerves were intact and functioning (demonstrated by pinching the snout region) in place of both naso-ciliary nerves as in the previous experiment. Autopsy revealed both maxillary nerves intact, while both naso-ciliary and all of the olfactory fibers had been severed.

For control, air blown into both nostrils, as shown by figure 2, row A, caused no change in the upper respiratory tracing or the lower blood pressure tracing.

The graph in row B, figure 2, was obtained at the time of insufflation of xylol into both nostrils for a short period. As marked changes are disclosed in the respiratory tracing at the top and the blood pressure tracing at the bottom as usually occur in records where all divisions of the trigeminal nerve are intact. Respiration is completely arrested and a sneeze reflex is induced. The normal depth of the respiratory excursions returns soon after insufflation ceases, but the rate does not become normal until 15 seconds after insufflation ceased. Blood pressure rose 25 mm. during stimulation and has not quite returned to normal at the end of this tracing. The rate of the pulse before stimulation was 8 beats in two seconds and during stimulation it dropped to 7 in two seconds, while the pulse waves were 1.5 mm. high before stimulation and 3 mm. high during stimulation.

Other xylol and benzol graphs were essentially the same as the graph reproduced.

Similar results were also obtained in repeating this experiment in rabbit (615) with the substitution of ammonia for xylol. During stimulation respiration was brought to a standstill, the blood pressure increased 21 mm., the pulse dropped one beat in 2 seconds and the height of the pulse waves increased 1 mm. during the interval of stimulation.

It seemed unnecessary in this experiment to sever the maxillary nerves and give insufflations with all three nerves sectioned since this had been done in several animals with negative results in the previous experiment, and negative results were also obtained from inhalation of these vapors in somewhat similar experiments to follow.

Direct effect of inhalation of xylol and weak ammonia vapor on the nerve endings of one maxillary nerve. The preparation of rabbit (619) for this

experiment consisted in severing both olfactory tracts, both naso-ciliary nerves, and the right maxillary nerve, so that the left maxillary nerve was probably the only nerve supplying the nasal mucosa left intact. Pinching both nostrils disclosed that the left responded but the right did not. Autopsy revealed all of the above nerves severed and the left maxillary intact. Blood pressure and thoracic respiratory records were obtained as before.

After closing the right nostril and holding a cone saturated with xylol over the left nostril (one having its maxillary nerve intact) changes in respiration and circulation appeared in these tracings which were only slightly weaker than those obtained in the previous experiment where both maxillary nerves were intact and were stimulated by the insufflation method. This record is not shown, but during inhalation it reveals respiration brought to a standstill, a blood pressure rise of 10 mm., a decrease in pulse rate of one beat in 4 seconds, and an increase in the height of the pulse waves of 1.5 mm.

The next phase of the experiment consisted in closing the left nostril and taking respiratory and blood pressure tracings during the time a cone saturated with xylol was held over the right nostril (one in which the maxillary nerve was severed in addition to the naso-ciliary and olfactory nerves). Tabulated data from this record conclusively show that no change takes place in respiration, blood pressure or pulse during the time of stimulation.

This experiment was repeated in rabbit 579 with identical results in using weak ammonia for the inhalant.

In this animal the experiment was carried one step further. The vagi, depressors, and cervical sympathetics were severed at the level of the larynx, and the same tests were repeated, but using very strong ammonia vapor for the inhalant. With this modification there could be no question but that the very strong ammonia vapor would bring about a trigeminal reaction if any trigeminal fibers were present, and if all were severed, the inhalation of this vapor into the lungs would not produce a vagus response. The results were as before, positive from the nostril in which the maxillary nerve was intact and negative from the one in which it had been sectioned.

Direct effect of inhalation of xylol and weak ammonia vapor on the nerve endings of one naso-ciliary nerve. This experiment is a companion to the previous experiment as to procedure, excepting that the left naso-ciliary nerve was left intact in place of the left maxillary nerve. Respiratory and blood pressure tracings were obtained at the time of inhalation of xylol through the nostril (left) in which the naso-ciliary nerve was intact resulted in very weak respiratory and blood pressure changes. There was some depression and inhibition of the respiratory excursions, a blood pressure

rise of 7 mm., a change in the rate of the pulse waves from 8 in two seconds to 7.5, but no change was manifest in the strength of the pulse.

A graph taken during the inhalation of xylol into the nostril in which the naso-ciliary fibers were cut in addition to the maxillary and olfactory discloses absolutely no alteration in respiration or circulation.

As a further step in this experiment the vagi, depressors, and cervical sympathetics were sectioned at the level of the larynx and the previous tests were repeated, using very strong ammonia vapor for the inhalant. The results were practically the same as when the vagus groups of nerves were intact and a weak ammonia inhalant was used. All records show no variation in respiration or circulation during inhalation of ammonia through the nostril in which all three nerves were severed.

This experiment was repeated in rabbit 573 and identical results were obtained.

It should be mentioned that Sandmann and others have obtained respiratory reflexes from faradic stimulation of various branches of the trigeminal nerve.

SUMMARY AND CONCLUSIONS

The direct effect of insufflation or inhalation of an irritating vapor on the endings of the maxillary nerves causes practically the same alteration of respiration and circulation as if both trigeminal nerves were intact. It consists of a complete suppression of respiration, usually terminating in a sneeze, a marked rise in blood pressure, and a slowing and strengthening of the carotid pulse.

The effect of the same stimulation on the naso-ciliary nerves is very much weaker. Respiration shows only moderate or slight depression and inhibition.

It is clear from the last two experiments that the ordinary vagus reaction of coughing and fall in blood pressure is not evoked for a considerable period of time during inhalation of xylol or weak ammonia vapor if the animal has the protection of one maxillary or one naso-ciliary nerve.

It is apparent from the insufflation experiments where the olfactory fibers were cut and the vagus groups were functionally eliminated by tracheotomy and from the inhalation experiments where the olfactory and vagus groups were sectioned, that the maxillary and naso-ciliary divisions of the trigeminus were the only nerves concerned with the conduction of impulses affecting respiration and circulation.

There seems to be no question but that sectioning the naso-ciliary and maxillary divisions of the trigeminus is as effective technique in producing the so-called *olfactory animal* as is the severance of both trigeminal roots intracranially.

The last two experiments also show that there is no crossing of the trigeminal fibers to the opposite nasal cavity or at least insufficient decussation to pick up an impulse from xylol or ammonia stimulation.

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DIRECTIONAL DIFFERENCES IN THE CONDUCTION OF THE IMPULSE THROUGH HEART MUSCLE AND THEIR POSSIBLE RELATION TO EXTRASYSTOLIC AND FIBRILLARY CONTRACTIONS[1]

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Heart muscle ordinarily conducts the wave of excitation with equal facility in either direction. This has seemed to be a necessary consequence of the myogenic theory which is now almost universally accepted. Nevertheless, it is well known that there may be differences in the duration of the pause at the auriculo-ventricular junction depending upon the direction of conduction, and that various forms of treatment of that region may variously affect the delay in the two directions, even to the extent of producing unidirectional block (Engelmann, 1894; Mines, 1914; Skramlik, 1920). Furthermore, it has been demonstrated that strips of heart muscle, apparently homogeneous, can be made to exhibit not only differences in conductivity depending upon the direction of the beat, but even unidirectional conductivity (Erlanger, 1906). The present experiments were carried out primarily in the hope of shedding some light on the conditions determining the development and the direction of such differences in conductivity.

The method employed has consisted, in principle, of exposing to different conditions contiguous stretches of a strip of ventricular muscle of the turtle, *Pseudemys concinna*, made to beat rhythmically in either direction by artificial stimulation of one or the other end. The details of the method have been described in connection with a search for satisfactory means of depressing conduction in turtle heart muscle (Schmitt, 1928). Various combinations of chambers with partitioning rubber curtains were arranged along the strips. There were always the two end glass chambers and one rubber curtain between them; but in the later experiments the muscle passed through, in addition, one, two or three intermediate hard rubber cells, entailing the use of two, three or four partitioning curtains, respectively. These were used in combination with the various local depressants,

[1] The experimental data in this paper are taken from the dissertation presented by Francis O. Schmitt in partial fulfilment of the requirements for the degree of Doctor of Philosophy, Washington University, 1927.

such as electrical polarization, cold and alteration in ionic balance. Resulting from these various treatments of the strip during the course of over fifty experiments, many instances of directional differences in conductivity and of other peculiarities were observed.

In order to facilitate presentation of the relevant data thus accumulated, certain terms will be employed which may be defined here. When the conduction rate through the injured segment of muscle is the same in either direction the state is said to be *isodromic;* when the rate is faster in one direction than in the other, the condition is said to be *heterodromic.* A *monodromic* muscle is one in which the impulse is transmitted in one direction but is blocked in the other; and it is *adromic,* that is to say, the block is complete, when the impulse can get through in neither direction. Under certain circumstances a second response of one side may follow the response of the other side in the absence of a second artificial stimulation. This return, or reëntry, of the impulse is made possible when the muscle is in the *opisthodromic* state. The corresponding substantives are *isodromia, heterodromia, monodromia,* etc.

In previous attempts it has been shown (Erlanger, 1906) that mere compression of a strip in a Gaskell clamp may be sufficient to develop directional differences in conduction. In the present experiments each of the rubber curtains exerts some degree of local compression which varied somewhat from preparation to preparation, depending upon the snugness of the fit. The seat of much of the delay in conduction from segment to segment, and of the directional differences in conductivity, was under the rubber curtains and might have resulted merely from the compression they exerted. With the simplest additional treatment, namely, two chambers (one containing a depressant) and one curtain, the impulse, depending upon the direction in which it is moving, passes either from depressed muscle, through a thin disc still more depressed, into normal muscle, or through a sequence the reverse of this. The problem was to produce directional differences in conduction and to determine whether there is any relation between the direction of the heterodromia or monodromia and the sequence of contiguous reactivities. The presence of additional chambers and partitioning curtains probably does not alter the fundamental conditions, but merely increases the chances of eliciting directional differences in conductivity.

It is realized that the method does not accomplish ideally the main end, namely, the division of the strip into contiguous stretches differing sharply from each other in respect to their reactivites. In the first place, as has been stated, the two regions are separated by a disc of tissue whose reactivity is depressed by the compression exerted by the rubber curtain. This compressed region, furthermore, will probably be depressed more by the action of the depressants used than other parts of the strip exposed to them, because, as Clark (1913) has emphasized, the more hypodynamic

the condition of the muscle, the more susceptible it becomes to the action of the reagents. Then, when KCl, for example, is added on one side of the curtain, it penetrates into the muscle of that side and presumably diffuses, though very slowly, into the normal tissue of the other side. This diffusion zone must take the form, roughly, of a cone whose apex protrudes more and more with time into the normal tissue. When the depressant is withdrawn this cone must recede in like manner. The action of the depressant, therefore, is not wholly confined to the side to which it is added. And, finally, it is well known that the response of heart muscle depends to a large extent upon the history of the tissue. In keeping with this it has been found in the present experiments that a second or third treatment with excess KCl, for example, produces effects that are more severe than the first, even when the concentration of the depressant is the same in each trial.

RESULTS. After a ventricular strip has been prepared in the manner previously described (Schmitt, 1928), the readings during the first few minutes usually reveal a slight degree of varying heterodromia; the conduction quotients (i.e., the time in one direction over the time in the other direction) are not constant. This is doubtless occasioned by the residual damage resulting from the manipulation of the strip. As the muscle becomes more normal, the conduction quotients from one period to the next reveal little if any variation from constancy. If, now, one end or the middle segment of the muscle be depressed by pressure, electrical polarization, or by chemical means, the conduction may thereby be much more affected in one direction than in the other, the degree of the difference depending presumably in some way upon the severity of the depression and the reactivity of the muscle.

By way of example, table 1 shows the development of heterodromia following depression by an excess of KCl in an experiment in which four chambers were used. The KCl content of chambers *1* and *2* here was increased temporarily, while the segments in chambers *3* and *5* remained in normal Ringer solution. And table 2 shows the results of an experiment in which the calcium was withdrawn from the Ringer solution of one side. It is seen here that a heterodromic condition rapidly passes over into monodromia during the treatment. The rapidity of the change was doubtless due to the fact that the strip had been previously depressed by an excess of potassium.

Here it may be pointed out that evidences of a fluctuating heterodromia are to be seen in Drury's (1926) experiments, also. This investigator studied the effect of uniformly applied pressure upon conduction in both directions through the auricle of the dog's heart. He concluded that "the general character of the block is identical whether the waves are travelling to or fro through the compressed muscle." Table 3, however, which we

have constructed from his data, reveals that not only does the degree of decrement vary depending upon the direction the wave is travelling, the difference being as much as 37 per cent, but also that this heterodromia fluctuates.

The condition of monodromia has been observed in muscle exposed to compression alone, and to compression plus either electrical polarization or chemical action.

TABLE 1

TIME	*1–5*	*5–1*	$\frac{1-5}{5-1}$	REMARKS
	seconds	*seconds*		
12:50	0.55	0.53	1.04	Normal Ringer in all chambers
1:00	0.53	0.53	1.00	
1:06	0.53	0.58	0.91	
1:12	0.58	0.55	1.06	←Ringer plus 0.07 per cent KCl in *1* and *2*
1:20	0.62	0.66	0.94	
1:25	0.63	0.68	0.93	
1:30	0.63	0.75	0.84	
1:33	0.63	0.82	0.77	
1:36	0.56	0.85	0.66	←Normal Ringer in all chambers
1:43	0.74	0.81	0.91	
1:50	0.80	0.80	1.00	
1:54	0.86	0.87	0.98	
2:01	0.90	0.96	0.94	←Ringer plus 0.07 per cent KCl in *1* and *2*
2:07	0.85	0.98	0.87	
2:14	0.90	1.06	0.85	
2:19	0.87	1.14	0.76	←Normal Ringer in all chambers
2:24	0.94	1.14	0.80	
2:35	0.98	1.09	0.90	
2:42	1.03	1.16	0.89	
2:54	1.12	1.29	0.87	←Ringer plus 0.07 per cent KCl in *1* and *2*
3:00	1.27	1.48	0.86	
3:05	1.05	1.68	0.63	
3:18	1.69	2.02	0.83	
3:22	1.70	2.21	0.77	←Normal Ringer in all chambers
3:30	2.00	1.86	1.08	
3:35	1.93	1.87	1.03	

The muscle was contained in four chambers (*1*, *2*, *3* and *5*), treadles *1* and *5* recording the contraction of the segments of the muscle in chambers *1* and *5*, respectively.

Monodromia by compression. In confirmation of Erlanger (1906) we have frequently observed in the present experiments during recovery from complete pressure block a stage during which the block is partial or even complete in one direction while the sequence is one to one in the other direction. An instance of such unidirectional conduction is seen in figure 1.

In many cases the very first impulse sent through in the better conducting direction may be conducted with the lowest grade of block of the stage of

TABLE 2

TIME	1-5	5-1	$\frac{1-5}{5-1}$	REMARKS
	seconds	*seconds*		
4:31	0.53	0.72	0.66	Normal Ringer in *1* and *5*
6:23	0.69	0.95	0.70	
6:57	0.64	0.93	0.66	
7:10	0.71	1.17	0.58	
7:13				Ringer minus calcium in *1*
7:18	0.71	1.36	0.48	
7:23	0.73	2.01	0.34	
7:28	0.74	Block		
7:33	0.77	Block*		
7:43	0.81	Block		
7:48	0.80	Block		

The muscle is contained in but the two chambers, *1* and *5*.

TABLE 3

STIMULATING	P-M OR M-P	M-D OR D-M	P-D OR D-P	PER CENT DIFFERENCE
	second	*second*	*second*	
Proximally	0.0043	0.0087	0.0130	13
Distally	0.0059	0.0088	0.0147	
Proximally	0.0046	0.0073	0.0119	
Distally	0.0078	0.0085	0.0163	37
Proximally	0.0059	0.0075	0.0134	
Distally	0.0069	0.0085	0.0154	15
Proximally	0.0054	0.0098	0.0152	
Distally	0.0062	0.0101	0.0163	7
Proximally	0.0068	0.0099	0.0167	
Distally	0.0061	0.0097	0.0158	−5
Proximally	0.0060	0.0085	0.0145	
Distally	0.0070	0.0107	0.0177	22

Data in the first three columns are taken from table IV of Drury's paper. P-D and D-P are conduction intervals between the extreme leads in either direction as indicated (P is the proximal, D, the distal, and M, the middle lead). The differences between P-D and D-P in per cent of the former are given in the last column.

the experiment then obtaining; and a partial one-way block may persist for many minutes. This observation rules out the possibility of explaining the

phenomenon on the basis of a *treppe* in irritability of the partially blocked side. In the stages pictured in figure 1, conduction was completely blocked for a while in both directions; then a *1–1* sequence developed in one direction without the intervention of any of the intermediate grades of partial block, although conduction in the reverse direction was still completely blocked.

Impairment following electrical polarization. A monodromic condition was noted several times developing immediately subsequent to the passage of strong polarizing currents through the strip mounted in the usual manner. Figure 2 shows a record obtained under such conditions near the close of an experiment in which the strip has been exposed to low current

Fig. 1. Monodromia following pressure impairment. Top line shows contraction of muscle in chamber *5*, second line, that in chamber *1;* third line signals the stimulating induction shocks; time, on lowest line, is in seconds. The record begins a few minutes after mechanical compression of the muscle, the direction of conduction, as indicated by arrow, *a*, being from *5* to *1*. After three beats the block in this direction becomes complete. *a*, *b*, and *c* show that the block is complete in both directions; *d*, *e*, and *f* show a unidirectionally complete block, the strip beating from *1* to *5* but not from *5* to *1*. This record shows that a tendency towards monodromia may develop within 50 seconds after the condition was one of total block.

strengths; at this time, however, a high voltage (5 volts) was being used. Despite this high potential, and despite the fact that the muscle was being stimulated at a maximal rate, normal conduction persisted. But at the moment the circuit was opened block supervened. This block, however, was unidirectional; it disappeared completely, and perfect sequence returned immediately, when the direction of conduction was reversed, and was complete upon returning to the original direction of stimulation. Figure 2 shows the reversal thrice repeated. The monodromia observed following the application of strong polarizing currents has had no apparent relation to the direction of the polarization. Whether the compression exerted by the curtain is of any significance in determining this effect of polarizing currents, it has been impossible to ascertain.

Fig. 2. Monodromia following polarization by a strong current. Coincident with, and therefore in consequence of, the opening of the polarizing circuit (indicated by rise of upper line) a high grade of partial block in the *1–5* direction appears while the sequence remains 1–1 in the *5–1* direction. Arrows indicate the direction of conduction. The lowest line signals the induction shocks by which the strip was stimulated, the rate being about 12 per minute. The time line is not reproduced. Greatly reduced.

Impairment by chemical means. Monodromia has been observed in many instances following one-sided chemical action. Table 2, already referred to, shows a case in which a monodromic condition was established following the withdrawal of calcium from the Ringer solution bathing one part of the muscle. The monodromia usually has made its appearance at a time when the conduction rate had been reduced greatly by the depressant; and it would seem that the relation between the reactivities of the muscle on the two sides of the curtain is a factor, as the following experiment indicates. A preparation, contained in four chambers, had been in use for some three hours and conduction had become very slow in both directions. Ringer solution containing 0.07 per cent KCl was then placed in chamber *2*. In 15 minutes, the impulse travelling from *2* to *1* was blocked, although conduction was still possible from *1* to *2*. In less than one minute, the block in the *2* to *1* direction disappeared, but two minutes later a block in the reverse direction became established; this block from *1* to *2* was permanent. Ten minutes later, segment *2* was again treated with an excess of KCl with the result that in five minutes, the impulse was completely blocked. Thirty minutes after restoring Ringer solution to segment *2* the block in the *2* to *1* direction disappeared, and, under these conditions, a unidirectional block persisted for many minutes. It has been impossible, however, to ascertain whether there is any definite relation between the development of monodromia and the sequence of the reactivities of the parts of the strips.

Heterodromia in relation to the sequence of irritability. Engelmann, in 1895, suggested that the phenomenon of irreciprocal conduction might be due to differences in the reaction characteristics of tissues. If one tissue requires a certain "specific energy" for stimulation and an adjoining tissue less, a unidirectional block may develop, in which the impulse passes from the latter to the former, but not in the reverse direction. Engelmann's quantitative experiments on irreciprocal conduction were done on single fibers of sartorius muscle, and on the basis of them he attempted to account for heterodromia and monodromia in heart muscle. Skramlik (1920) has called attention to a serious difficulty which the Engelmann hypothesis encounters when applied to the conducting system of the whole heart. In the frog heart, in the normal sequence of events, the impulse must pass from the fast conducting auricular fibers through the very slow conducting "block" fibers, into the relatively fast conducting ventricular fibers. If quantitative reaction characteristics play the decisive rôle, then in the normal A-V sequence the impulse must encounter at least one boundary where conditions are very unfavorable for further propagation. Englemann was very careful, however, to state (p. 281) that irreciprocal conduction must be thought of as being caused not only by quantitative factors of reactivity, but also by qualitative factors, the nature of which is entirely unknown.

In further work upon the nature of irreciprocal conduction, Engelmann (1896) studied conduction in the fibers of large sartorius muscles. The curarized muscles were suspended horizontally with the two ends separated by means of cotton padding soaked in Ringer solution, the muscle being transfixed at the mid-point by pins. Under terminal conditions of fatigue, or following local cooling, he succeeded in producing unidirectional block. In these experiments he sought to localize the effect to single fibers by making incisions into the muscle at the mid-point so that comparatively few fibers conducted the impulse from end to end, and used supramaximal stimuli so as to insure stimulation of all of the fibers still conducting, escape of stimulus across the mid-point being minimized by separating the two ends of the muscle by dielectrics. He was still able to demonstrate monodromia and he consequently concluded that the phenomenon can be exhibited by single fibers, and made the generalization that the impulse is conducted more easily from rapidly conducting tissues to slowly conducting tissues than in the reverse direction. Although his work is now more than thirty years old, we know of no attempts at verification. It might be pointed out that in his experiments, heterodromia was found to precede monodromia even in single fibers (p. 407). This, in the terms of his hypothesis, must mean that in the conduction of the impulse from one hypothetical tissue element to the next, the disparity in specific stimulating energies of the two elements manifests itself in a delay at the junction, provided that each element be considered uniform as regards its conduction properties. This phenomenon of building up of stimulatory energy at junctions at the expense of velocity of conduction is one concerning which little is known, even at this time. It is beyond the scope of this paper to discuss these fundamental questions, but the above considerations suffice to indicate some of the difficulties that confront the Engelmann hypothesis as applied to heterodromia in single fibers.

The experiments in which one side or one segment of the strip is treated with Ringer solution containing four to five times the usual quantity of KCl seemed to afford a means of putting the Engelmann hypothesis to the test. With this in view, the irritability of the muscle in the several chambers was followed by determining the thresholds to electrical stimuli. In 44 trials taken from 14 experiments in which these relative irritabilities were known, 27 indicated that conduction was slowed more when the impulse passed from a tissue of high irritability to one of lower irritability than in the opposite direction, 8 indicated the reverse, while 9 could not be placed in either category. The only conclusion warranted by these results is that there is no constant relation between the irritabilities of contiguous stretches of heart muscle and the conduction rate between them. Indeed, the figures, if anything, contradict the view sponsored by Engelmann, though, as will be made clear below, this is not the inevitable consequence of our results.

The significant result, then, seems to be the lack of any consistency in the direction of blocking relative to the treatment of the strip. A similar lack of consistency is to be noted in other investigations in this field. Engelmann, himself (1894, p. 163), found that sometimes the block in suspended heart preparations was of the A-V type, while at other times it was of the reverse type, although the conditions of the experiments were identical in all cases. Erlanger (1906) found that monodromia occurred under the conditions of his experiments, usually, but not invariably, when the impulse was forced through the strip in one direction over long periods of time. Mines (1914), likewise, noted inconsistencies in results. He says, "in some cases the direction blocked was from the ventricle to auricle, in other cases from auricle to ventricle." The data of Drury (1925), already referred to, show, upon analysis, a similar variability in the direction of heterodromia when the depression is produced by mechanical compression.

Discussion. In view of these inconsistencies in the direction of heterodromia and of monodromia, it seems more reasonable to explain the results of our experiments on a morphological basis. We postulate the existence of multiple conducting pathways in the strip, some conducting better in one direction, some in the other. Upon this basis, heterodromia and monodromia receive a ready explanation if it be assumed that under the influence of depressants and of compression either separately or together, one of these paths is affected later or less than the others and determines the observed conduction characteristics. In all probability the irregular heterodromia observable immediately after the preparation of the strip also is indicative of the existence in the strip of pathways conducting with different degrees of heterodromia, which, owing to the severe treatment it receives, are not the same in the successive contraction cycles. The histology of cardiac tissue, with its multiple branching pathways, is not inconsistent with such an interpretation. Garrey (1914), it may here be recalled, has directed attention to the necessity of assuming local monodromia in ventricular muscle in order to account for the production of circus movements. Skramlik (1920), furthermore, has attempted to explain certain auriculoventricular monodromias by means of a similar hypothesis. (He found that if all parts of the A-V junction of the frog's heart be cut with the exception of the narrow dorsal band, or if all parts but this band be cooled, conduction from A to V alone was possible; that, on the other hand, if only the septum be allowed to remain intact, only V-A conduction was possible.) He was unable, however, to detect monodromia in the continuity of the ventricular musculature. Skramlik, while admitting in his discussion that his work does not supply the information necessary to ascertain the fundamental nature of monodromia, expresses the opinion that it strengthens the view that conduction in heart muscle may be conditioned by some sort of nervous mechanism. But the present observations, and those

of Erlanger (1906), also, demonstrating that the monodromic condition may develop in ventricular strips, would seem to preclude a neurogenic explanation.

If our results are best explained by assuming pathways in ventricular muscle that conduct better in one direction than in the other, it might follow that they throw relatively little light on the question of the relation of reaction characteristics of contiguous stretches of heart muscle to the transmission of the impulse. For there is always the possibility that the characteristics of the path dominant at the moment so strongly favor a given heterodromia that a chemical treatment calculated to reverse the characteristics of the contiguous stretches that are determining the heterodromia does not suffice to completely overcome the predominant direction of conduction. There is also always the possiblity that the particular locus in the path that is determing the one-way conduction may lie at a point that feels relatively slightly the differential action of the treatment to which the two sides of the strip are exposed. To the extent to which this can happen, the method is inadequate as a means of establishing the relation between tissue reactivity and the direction of more ready conduction. Nevertheless, the fact that in over 60 per cent of the cases the delay was greater when propagation was from the more irritable to the less irritable side, if of any significance whatever, is opposed to the view that conduction is facilitated by this particular relationship of tissue characteristics. In any event, it seems fair to assume that there are pathways in the ventricular musculature, which, under normal conditions conduct better in one direction than in the other.

THEORETICAL. The present experiments throw no light whatever on the fundamental mechanism of heterodromia and monodromia. Engelmann's attempt to account for the phenomena has already been referred to, and nothing further need be said in regard to it other than to repeat that he believed (1896) that they could develop in single fibers. This is equivalent to maintaining in the case of heart muscle, that conduction along the syncytial fibers is not necessarily reciprocal. If so, some device must be hypothecated for the production of this state of affairs other than that which obtains at a nerve synapse, for instance, with its break in direct continuity. Mines (1914) has suggested that "the cause of unidirectional block (at the A-V junction) may very likely be expressed in terms of Adrian's work. If the decrement is uniform, then the system is symmetrical, and the block should be equal in the two directions. But if the decrement is greater in one end of the depressed region than in the other, we may have the possibility that the transmission in the one direction may be easier than in the other." Though doubt has been cast on the existence of decremental conduction in nerve (see for example, Kato, 1926; and Davis, Forbes, Brunswick and Hopkins, 1926), there is, nevertheless, some

evidence indicating the possibility of decremental conduction in heart muscle when injured (Drury, 1926; Schmitt, 1928). Therefore, although the Mines theory is expressed in but the few words quoted, and is based only upon the fact of A-V monodromia, it seems worthy of further develop-

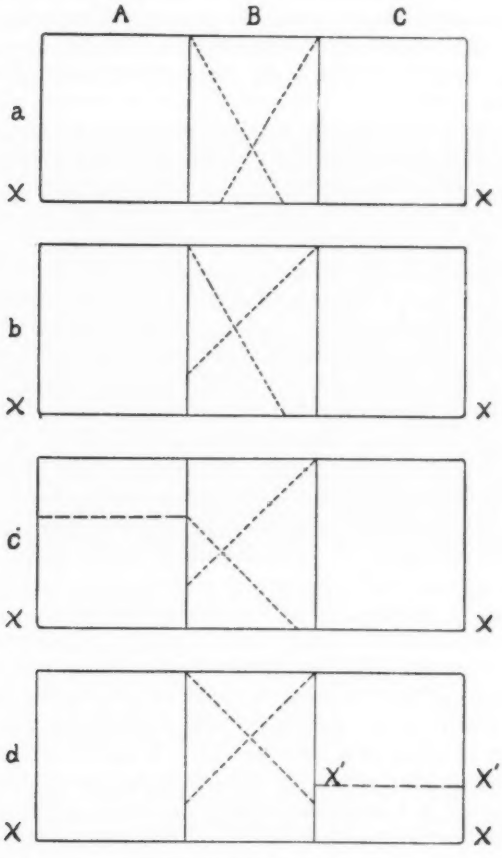

Fig. 3

ment. We may imagine that it finds expression in the diagrams *a* and *b* of figure 3. In these illustrative diagrams, segment *B* is a region of depression in which decremental conduction obtains between two untreated, normally conducting segments, *A* and *C*. The lower lines, *XX*, represent the threshold of stimulation to which the tissue will respond, the upper

horizontal lines, the normal impulse strength, and the dotted lines, impulses of subnormal or decrementing strength. Although the impulse in some instances is pictured as dying out in the depressed region, it may be supposed that it might actually reach the junction, but, being subminimal relative to the next segment, be ineffective. Diagram *a* represents complete block; the decrement is uniform and the system is symmetrical. Diagram *b* represents a case in which the decrement in one direction is greater than in the other, the condition which, in the opinion of Mines, might make unidirectional block possible.

This conception attributes the monodromia to characteristics of the depressed tissue alone, the reactivity of segments *A* and *C* being left out of consideration. It is possible, however, on the basis of decrement, to account for monodromia without assuming that the muscle in the region of the depression has acquired this asymmetrical action on the impulse. Diagrams *c* and *d* are constructed on the assumption that the decrement in segment *B* is the same in both directions so long as the strength of the impulses entering the region is the same. If the strength of the impulse coming from *A* is less than that coming from *C*, a unidirectional block might result as shown in diagram *c*. To this might be added another factor brought out by Schmitt (1928), according to which the actual slope of the gradient would be steepened if the strength of the initial impulse is weakened; this factor would tend to make monodromia even more likely. Again, if the strengths of the impulses from each side are identical but the threshold of segment *C* raised from the level *XX* to *X'X'*, monodromia might result as shown in diagram *d*. This hypothesis seems adequate to account for A-V monodromia without assuming a fundamentally asymmetric decremental state of the fibers of the A-V conducting system.

Opisthodromic action—Reëntry phenomena. In eleven of the later experiments, in which more than three chambers were used, necessitating the use of three or four curtains, a group of phenomena frequently developed which will now be described. The simplest case will be discussed first; it is one in which four chambers (*1*, *2*, *3* and *5*) and three curtains were used, the middle curtain, by chance, being the tightest fitting. It will be illustrated by a specific example taken from experiment 40. This experiment had been under way for over four hours. The procedure had been to vary the KCl content of the Ringer solution in chambers *1* and *2*, the contents of chambers *3* and *5* remaining normal Ringer solution throughout. Observations relevant to the present topic began at a time when segments *1* and *2* were depressed by KCl. Conduction had fallen to a very slow rate, especially under the curtains, but the depression was not symmetrical; in the *1–5* direction the rate was reduced over 70 per cent more than in the *5–1* direction. Analysis of the partial intervals showed that the point of the greatest delay was at the middle curtain between chambers *2*

and *3* which happened to be the tightest fitting. Here the conduction was exactly 100 per cent faster in the *5–1* direction than in the *1–5* direction. At this time an impulse was sent through in the *1–5* direction, which, after a delay of 2.58 seconds under the middle curtain, elicited successive contractions of the segments in chambers *3* and *5*. Immediately a return wave, starting spontaneously, caused a second contraction of segments *2* and *1* in turn. An example of such a return of the stimulus at a later stage of the same experiment is shown in figure 4. The phenomenon developed at a time when the impulse in the *1–5* direction was rapidly going over into

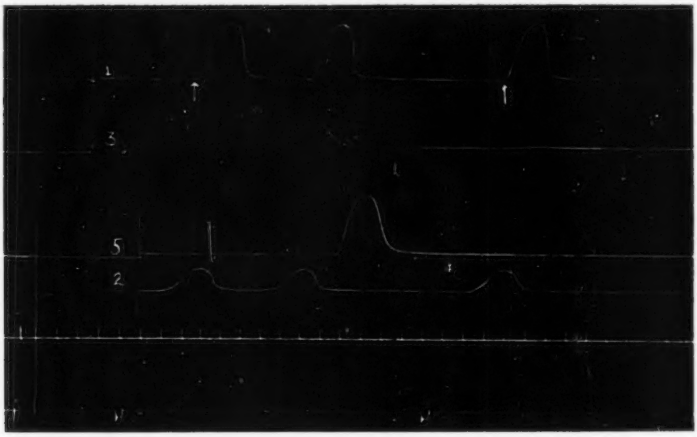

Fig. 4. Reëntry phenomenon. The muscle runs through four chambers (*1*, *2*, *3*, *5*), the contractions in each being registered by the corresponding treadles as numbered. Time is in seconds; moments of stimulation are recorded on the lowermost line. The first stimulation of *1*, indicated by the first arrow, calls forth a wave of contraction which is quickly followed by a reëntrant wave. The next impulse, indicated by the second arrow, caused by a second stimulation of *1*, is blocked between chambers *2* and *3*. Note the relative positions of the zero points.

block. Thus when *1* was stimulated a second time, the impulse, as figure 4 shows, was actually blocked, though the speed of conduction in the *5–1* direction was still fairly high.

In general it may be said that this phenomenon, which is essentially so-called reëntry, makes its appearance at a time when there is a condition of heterodromia bordering very closely upon monodromia; and it usually lasts only a few minutes, though it has occasionally persisted many. The reëntry seemed most likely to develop after one end of the muscle was suddenly and profoundly altered, as, for example, after one side, having been greatly depressed by KCl poisoning, was treated again with normal

Ringer solution, and when, under such circumstances, the direction of conduction was from the poisoned towards the untreated side, though these were by no means the conditions always obtaining.

In seeking an explanation of this phenomenon, two alternative possibilities suggest themselves: the impulse on its round trip either retraces its path or circles back. The former view leads to difficulties insurmountable in the present state of our knowledge; on the other hand, if it be assumed that the impulse travels over different pathways on entering and reëntering the depressed region, a simple explanation in accord with the facts can be offered. It will be convenient, in developing this view, to illustrate by the conditions and data drawn from experiment 40 (see fig. 4). Figure 5 is a diagram representing in simplest form the essential conditions of this experiment. *A* and *B* are two strata of fibers or two fiber paths passing through a curtain applied at *M*. Under the curtain, the fibers have been subjected both to pressure injury and to KCl poisoning, and as a result of

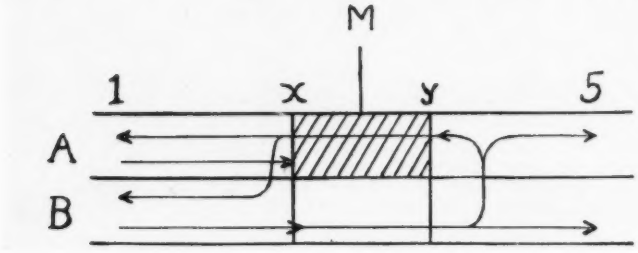

Fig. 5

this treatment, the segment between *X* and *Y* is the seat of greatly slowed conduction in both directions. Stratum *A*, being more directly exposed to the compression exerted by the curtain, has been more seriously affected than stratum *B* with the result that a monodromic condition has been produced in the former. An impulse originating at *1* and travelling towards *5* starts, presumably with the same conduction rate, in both *A* and *B*. But when the impulse in *A* arrives at *X*, it is blocked, owing to the monodromic condition of this section of *A*, and dies out. The impulse in *B*, however, traverses the region *XY*, but at a very slow rate. The pause in this case was 2.50 seconds! Upon leaving the region of depression at *Y*, the impulse in *B* spreads out according to the v. Kries (1913) principle of unlimited auxomerie, possibly by means of anastomoses, and goes forward now in both *A* and *B* and causes the segments beyond to contract. At the same time, however, the impulse turns back in *A*, passes through segment *YX*, which, not having contracted at all, is irritable, and causes segment *1* to contract a second time. This sequence is made possible first, by the

greater facility with which the impulse enters the region *YX* from *5* than from *1* as indicated by the conduction times through the strip in either direction, namely, 2.86 seconds from *1* to *5* and 1.43 seconds from *5* to *1*. It is made possible also by the sufficiency of the interval of time elapsing between the initial and the final contractions of segment *1*, an interval that would include the passage of the impulse through the *XY* segment both going, which in this particular case is 2.5 seconds, and returning. There are good reasons for maintaining that this time exceeds the refractory period of segment *1*. Thus the systolic time, which may be taken as a rough measure of the refractory period, had a duration of 1.5 seconds. And, furthermore, it was observed that a brief exposure of this strip to 0.025 per cent solution of $BaCl_2$ caused a series of contractions with a period of 2.0 seconds, which

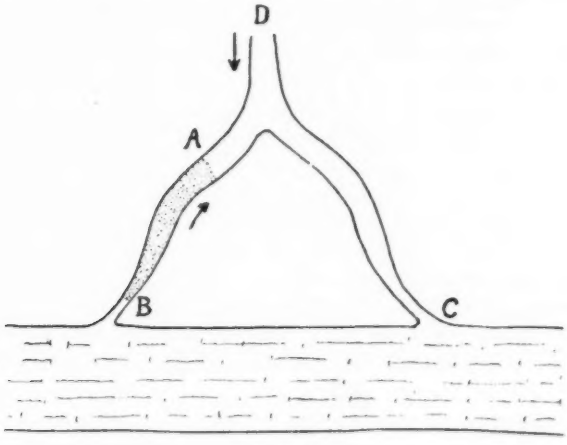

Fig. 6

must be somewhat longer than the absolute refractory period. To recapitulate then, the conditions that make possible a reëntrant beat under the circumstances obtaining in this type of experiment are *a*, monodromic conduction in *A* in the *5–1* direction, and *b*, a delay in the transmission of the impulse from *1–5* in *B* sufficient in duration to permit *A* (and also *B*) in segment *1* to recover from refractoriness.

Bearing upon ventricular extrasystoles. This analysis of the conditions determining reëntry in strips suggests a simple explanation of spontaneous ventricular extrasystoles of the coupled type in the mammalian heart, based upon the arrangement of the ventricular conduction system. In figure 6, a penultimate twig, *D*, of the A-V bundle is shown dividing into two terminal branches which anastomose with ventricular muscle fibers at *B* and *C*.

Under normal conditions, the impulse from *D* reaches *B* and *C* at approximately the same time, throwing the ventricular musculature at these points into contraction at almost the same instant. But if one of the two terminal branches happened to be the site of a local lesion, or of some other state that depressed conduction in it, the conditions might very well develop which experimentally lead to a reëntrant response. It merely would be necessary that the impaired segment between *A* and *B* become monodromic with conduction impossible in the *A-V* direction but still fairly good in the reverse direction. Then an impulse coming from *D* would be blocked at *A* and would die out, but by way of the other terminal branch it would reach and stimulate the ventricular musculature at *C*. The excitation from the ventricular fibers would then reënter the Purkinje system at *B* and traverse the region of injury, but at so slow a rate that by the time it arrived at *A*, the uninjured fibers there would have recovered from refractoriness and would again be excited. This excitation would immeditely spread through the conducting system and reëxcite the entire ventricular musculature. The next normal impulse from the auricle would then supervene to stop this circus movement by rendering the normal fibers refractory. That there could elapse an interval sufficient in duration for the recovery of *A* from refractoriness, as required by this hypothesis, is indicated by many published records of greatly prolonged *A-V* pauses in man. Barker and Bridgman (1917), for instance, cite cases in which A-V pauses as long as 0.88 and 1.03 second were recorded.

This conception of the origin of extrasystoles in the mammalian ventricles, it should be pointed out, is not inconsistent with existing electrocardiographic evidence indicating that spontaneous premature beats arise in the conducting system (Lewis, 1925, p. 387). It has the advantage, furthermore, of attributing a phenomenon, which may be almost normal in its incidence, to a lesion, or, it may be, to merely a temporary functional disturbance, in an ultimate and unessential branch of the conducting system. It might be pointed out, though, that a depression or a lesion at any point in the triangular circuit, *ABC*, of figure 6, or the development in any part of the musculature of a state of affairs comparable to that obtaining in our strip experiments, could cause a reëntrant wave by a similar succession of events. As has been said, however, evidence strongly favors the view that the phenomenon in the great majority of the cases develops in the conducting system.

Local circulating rhythms and associated phenomena. A slight modification of the conditions portrayed in figure 5 suffices to supply those needed for the development of a local circus. The impulse returning through *XY* from *5* traverses *B* to the left but fails to turn to the right in *1B* and traverse *B* and *5* again presumably because the time relations happen to be such that, though *B* in segment *1* has recovered its irritability, under *M* it has not, or because *B* at *M* conducts the impulse more readily to the

left than to the right and, under the conditions found by the impulse returning from *5*, is monodromic. But if the delay in the return of the impulse were slightly longer, or if the heterodromia were less marked or absent, *B* under *M* might also be stimulated by the returning impulse spreading into it from *A1*. Thus would be started a local circus. The part of *B* in segment *M* would escape stimulation by the impulse returning in *M* if (a) the time interval were not long enough for *B* there to recover its irritability and (b) if the subnormal impulse in *M* failed to reach the threshold of the relatively refractory *B* there. Thus the conditions be-

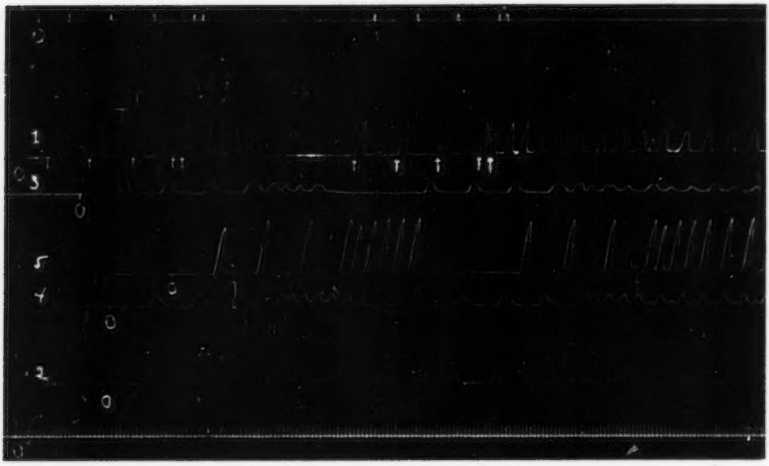

Fig. 7. Multiple response from an extra stimulus. Same conditions as in figure 8. Segment *1* was stimulated as indicated by the arrows. A series of beats follows an extra-systole after the fourth contraction. Two instances of this are shown. Zero points indicated. Reduced.

come clear under which a circus can develop in a comparatively limited locus.

The development of such a local, self-perpetuating circus, together with propagation of the impulse through the length of the strip with each circuit, would account for the responses portrayed in figures 7 and 8. The strip in these experiments passed through five chambers and four curtains. In the case shown as figure 7, four stimuli applied to segment *1* in succession resulted each in the conduction of just one impulse through the strip; but a stimulus thrown into segment *1* so as to produce an extra contraction immediately after the fourth contraction, called forth a long series of regular contractions arising spontaneously in segment *1* and passing thence through the length of the strip.

Stimulation of heart muscle at the end of the refractory period has long been known to induce repeated contractions, circus contractions and fibrillation. On the basis of the observations and views recorded in this paper what happens under such circumstances is that the stimulus is applied to, or passes into some one of the many pathways in the heart musculature that are potentially monodromic. It is obvious that nothing is better calculated to unfold such a monodromia than a second impulse following another early in its relatively refractory period. The possibility is thus provided of the establishment of a circus which may then persist for a

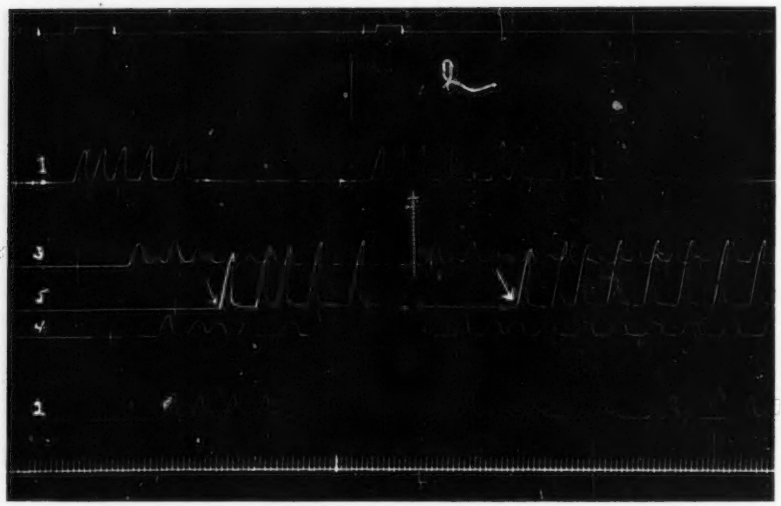

Fig. 8. Multiple response from a single stimulus. The muscle was contained in five chambers, the records being numbered accordingly. A rise in the top line indicates stimulation. Time is in seconds. At the first arrow, segment *5* was stimulated once. This called forth a series of beats without further artificial stimulation. A similar series resulted after a second stimulation, indicated by the second arrow. Reduced.

longer or shorter time. The result would be a tachycardia like that of figure 7.

In essentially the same way it is possible to account for the response pictured in figure 8. Here a single stimulus applied to segment *5* of the quiescent strip produces a series of contractions of the whole strip originating in *5* and causing the other segments to contract in turn. Should a part of the connection between segments *5* and *4* happen to be monodromic either at ordinary rates of stimulation or because of the depressed state that obtains during tissue inactivity, the conditions would be pro-

vided that could lead to the establishment of a circus there. The fact that the spontaneous contractions cease after a time favors the latter interpretation; they cease because the monodromic state disappears as, with activity, the reactivity of the strip rises.

Applications to circus movements in the intact heart. In the case of the intact ventricles a local circus produces the picture of fibrillation because, on the basis of our views, some of the rapidly recurring impulses arising from the local circus artificially started, when conducted into the various pathways in the musculature, are blocked, completely in some, monodromically in others, and, in the latter event, start other circuses which in turn act as centers for the distribution of additional rapidly repeated stimuli. The state of affairs thus produced would be quite like that originally visualized by Garrey (1914).

The excellent accounts of the course of the impulses in auricular flutter and fibrillation that have come from Lewis' laboratory would lead one to suppose that potentially monodromic pathways exist likewise in the walls of the auricles, especially in the vicinity of the mouths of the great veins. It may be assumed that here the monodromia that permits of the establishment of the local circus develops when the tissue (in the dog) is stimulated at the rate of about 350 to 500 per minute, and this circus then distributes impulses at about the same rate producing a tachycardia or flutter of about that rate in the outlying parts of the auricles.[2] At higher rates of stimulation the potentially monodromic radiating pathways, it may be assumed, become actually monodromic also, with the result that local circuses develop in them, and the condition of fibrillation is thus established throughout the auricular musculature.

Thus, it is seen, the mechanism concerned with the production of coupled extrasystoles, tachycardia and fibrillation, as has been suggested by others, may be fundamentally one. Pathways which are potentially or actually monodromic (and it is to the knowledge of the rôle of such pathways that this paper contributes) permit a local circus to develop which produces extrasystoles or tachycardia and flutter, or multiple circuses to develop and produce fibrillation.

[2] This, it should be noted, is not Lewis' conception of the inception of the circus. He holds (p. 320) that at rapid rates of stimulation a wave may find its progress barred in one direction. The reason for this, he says, is that "the muscle is verging on the half-rhythm, there are fibres or islets of tissue which fail to respond, and it may happen that they are at a given instant more concentrated in one limb of the circle than in the other. In such an instance the wave propagates itself along one limb only." On such a basis, however, it is difficult to account for the flutter and fibrillation that result from a single stimulus, and impossible to account for the monodromia of our strip experiments.

SUMMARY

1. Experimental conditions are described by means of which it often is possible to bring about readier conduction through a ventricular strip in one direction than in the other (*heterodromia*). When the treatment has been sufficiently severe the condition may go over into one of unidirectional block (*monodromia*).

2. Monodromia has appeared following depression by local compression, and, in association with compression, by the passage of strong polarizing currents, and by variation in the ionic balance of Ringer solution, locally applied. In the experiments with ionic imbalance there was no rule governing the direction of better conduction; in the majority of trials conduction was faster when the impulse passed from a region of relatively low irritability than when it passed in the opposite direction, though the reverse frequently obtained.

3. This result is interpreted as indicating that there are normally in the strips multiple pathways, some conducting better in one direction, some in the other, the direction of the heterodromia or monodromia being determined by the characteristics of the most resistant or the best protected of the pathways.

4. It is suggested that the Mines theory of auriculo-ventricular monodromia, namely, that the condition may be due to asymmetric decremental conduction in a depressed region, be made more general and modified so as to include strength of impulse and irritability of the responding side as additional variables.

5. In strips passing through multiple chambers and rubber curtains, the phenomenon of reëntry (*opisthodromia*) has been repeatedly observed. Conduction data presented are consistent with an explanation based on the assumption that a monodromic condition exists in some of the fibers or fiber paths, and a heterodromic condition in adjacent fibers in the region of the muscle under some one of the compressing curtains.

6. Based upon local monodromia in an ultimate branch of the conducting system, an explanation is offered of the mechanism of the coupled type of ventricular extrasystoles.

7. Finally, it is shown how a slight modification of the conditions that produce reëntry would lead to the establishment of local circus contractions, and the relation of circus contractions so produced to tachycardia, flutter and fibrillation is discussed.

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THE RELATION OF INITIAL VOLUME AND INITIAL PRESSURE TO THE DYNAMICS OF THE VENTRICULAR CONTRACTION

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The importance of the initial distention of the ventricular muscle in regulating the magnitude of the cardiac contraction has been definitely established. But it has not been determined whether this regulation is a direct function of the intraventricular pressure at the onset of contraction (initial pressure) or of the associated ventricular volume at this time (initial volume). Frank (1895) showed some time ago—and his work was confirmed by Kozawa (1915)—that, when the heart is distended progressively, the changes in initial volume are caused by changes in initial pressure; the relation between the two being more nearly that of a geometric progression rather than an arithmetric one. Starling, Patterson and Piper (1914), and subsequently Gesell (1916), claimed that initial pressure and volume could be dissociated in the mammalian heart under physiological conditions, and that under these circumstances the pressure development and systolic discharge of the ventricles depended on the initial volume. Straub (1914) by similar experiments maintained, on the contrary, that the activity of the ventricle was dependent on the initial pressure. Wiggers (1928) has held as a result of an extensive experience that under normal circumstances initial volume and pressure changes are not dissociated but always vary in the same direction. The only exceptions noted by Wiggers were in premature beats (1925) and following the administration of epinephrin (1927). In both cases the changes in cardiac activity paralleled the variations in initial volume and not those of initial pressure. These exceptions would seem to confirm Starling's view (1915) that the regulation is by initial volume and not by initial pressure. But the dissociation of initial pressure and volume which occurred in these experiments probably was caused by an alteration in the inherent properties of the cardiac muscle for which the epinephrin was responsible in one case (cf. Wiggers, 1925) and the incomplete recovery of the heart in the other. The modification of the inherent properties of the muscle might in itself be the cause of the change in cardiac activity or, at least, might ob-

scure the effects of initial pressure and volume changes. This criticism is probably also applicable to the experiments of Gesell (1916) and of Starling and his associates (1914).

A similar objection holds against Gesell's conclusion (1916) that both initial pressure and initial volume have a regulatory influence on cardiac activity. The spontaneous variations in tone of the turtle's auricle, which he employed to dissociate initial pressure and volume, introduce changes in the inherent properties of the muscle, and their effects, which are difficult to evaluate, as he himself appreciated. In fact, it led him in a later paper (1920) to contradict his former conclusion that an elevation in initial pressure is beneficial, and to assert that raising the initial pressure was detrimental to the heart.

Gesell (1916) also used another method, which did not depend on the variation of the inherent properties of the muscle, to dissociate the changes in initial volume and pressure. But, it is difficult to see how the arrangement which he used could cause such a dissociation. From his diagram of the apparatus it is apparent that any change in initial volume *was bound* to change the initial pressure in the same direction, because the fluid level in the tube surrounding the heart would vary (i.e., level *D* in tube *C* of fig. 1, 1916), unless this were counteracted by a change in the tone of the auricle. The introduction of tone changes was exactly what Gesell wished to avoid. His failure to observe changes in initial pressure probably does not indicate their absence but rather the inability of the insensitive manometer to detect them. Most of the manometers employed in the past have not always been capable of recording the changes in initial pressure either because they were too insensitive or had no base line check (cf. Wiggers, 1928). This fact, for example, may explain why Segall and Anrep (1926) failed to find any change in initial pressure accompanying the early increments of initial volume.

The problem of determining which factor regulates the magnitude of cardiac activity cannot be settled by comparing the relative magnitude of the initial pressure and volume changes at various stages of filling, as Kozawa (1915) maintained. Proof may, however, be obtained by dissociating the two factors while the other conditions, especially the inherent properties of the heart, are kept unaltered. The manometer employed in such studies must be sensitive enough to record the changes in initial pressure. Recently, Edwards and Cattell (1928) have shown by such a method that increasing the initial pressure by 60 atmospheres has a beneficial effect on a heart kept at a practically constant initial volume. It does not follow that smaller increases, of the order of magnitude encountered ordinarily in physiological experiments, will have a similar effect. Nor does it preclude, as they point out, the presence of a regulation by initial volume.

In the present research an attempt was made to dissociate initial pressure and volume by external means and determine the effects on the contraction of the heart. For various reasons, the experiments were made on the

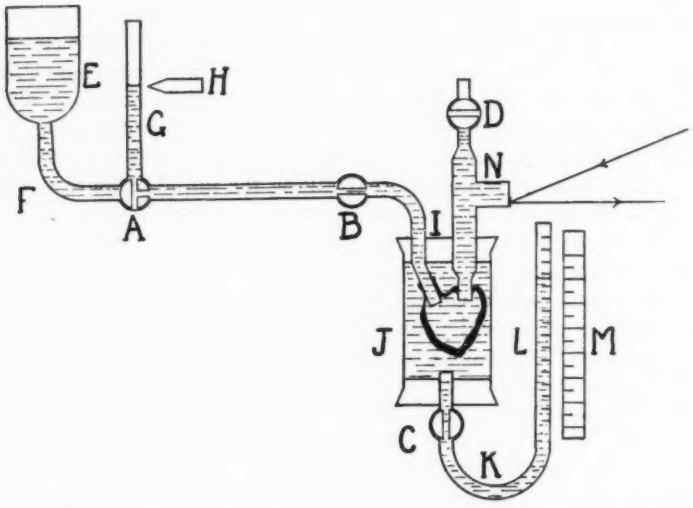


Fig. 1. Schematic diagram of apparatus used. The set up shows conditions during a so-called auxotonic contraction with the ventricle in systole. The turtle's ventricle is shown in cross-section immersed in the saline bath and attached to the manometer and perfusion cannulae. *A*, three-way stopcock for making various connections between saline reservoir, vertical tube and ventricular cavity. *B*, *C*, *D*, two-way stop-cocks. *E*, saline reservoir, whose vertical height is adjustable. *F*, flexible tubing. *G*, vertical tube with which ventricle is connected to make it contract auxotonically. *H*, marker used to check the constancy of initial pressure when the heart is contracting auxotonically and the initial volume alone varied. *I*, perfusion cannula, whose stopcock, *B*, is closed when recording isometric contractions and kept open during perfusion and when recording so-called auxotonic contractions. *J*, saline bath enclosing ventricle which is sealed and entirely free of air bubbles. *K*, flexible tubing. *L*, graduated 5 cc. pipette, adjustable vertically so that either the initial pressure or volume of saline bath, *J*, can be kept uniform. *M*, millimeter scale. *N*, optically recording manometer, the horizontal limb of which is covered at its end with a light rubber membrane on which is placed a mirror to reflect a beam of light into the camera (beams indicated by arrows). Further details of use of apparatus given in text.

isolated ventricle of large turtles. A procedure was developed whereby it was possible in such a rhythmically beating ventricle to have four combinations of changes in initial pressure and volume while the conditions of the heart were otherwise constant. The combinations were: 1, a con-

stant initial volume and varying initial pressure; 2, a constant initial pressure and varying initial volume; 3, the two changing in the same direction; and 4, in opposite directions. Most of the observations were made on the isometrically contracting ventricle where practically all the mechanical effort could be measured as pressure. A few observations were also made with the so-called auxotonically contracting ventricle.

METHOD. The apparatus used is shown diagrammatically in figure 1 and a brief description is given in its legend. The variations in the magnitude of contraction were determined from the pressure developed by the ventricle which was recorded with a modified Wiggers' manometer (*N* in fig. 1). The manometer was inserted into the ventricle via the a-v ring and the heart tied around the cannula on the ventricular side of the ring. The manometer differed from the one used in mammalian work in that the upright limb was shorter, the cannula was made of glass of 4 mm. bore instead of metal, and the recording membrane was more sensitive. At a meter distance 1 mm. in the record was equal to about 4 mm. of saline pressure. The vibration frequency of the manometer was less than that of the manometer used in mammalian work, but was adequate to record the slow changes in the turtle heart.

The perfusion of the turtle's heart, which was carried out only between one set of observations and the next, was accomplished by connecting the ventricle with an adjustable saline reservoir, *E*, by means of a perfusing cannula, *I*, thrust into the ventricle through the aorta. The fluid in the heart was allowed to drain out of the top of the ventricular manometer, *N*. During the course of a set of observations the stopcock, *D*, was turned and the manometer sealed at the top. Physiological saline was employed as a perfusate because the ventricles spontaneously beat in a regular manner in it.

The observations were made either on isometric or auxotonic contractions. The nature of contraction was regulated by suitable adjustment of stopcocks *A* and *B*. By turning stopcock *B* so as to seal the perfusing cannula, the contraction of the ventricle was made isometric. By turning stopcocks *A* and *B* so that the heart communicated with the vertical tube, *G*, the contraction was made auxotonic. The minute compression of the fluid, the movement of the manometer membrane and particularly the small amount of shortening in fractions of the ventricle prevented the contraction from being absolutely isometric in the former case. The term isometric will be used in the relative sense in this paper.

The dissociation of initial pressure and volume depended on the use of a liquid system not only within the ventricle but also around it. Care was therefore taken to have the ventricle, the manometer and the perfusing system free of air bubbles when the heart was attached to the cannulae, and none was permitted to enter during the course of the experiment. Similarly, care was taken to ensure the absence of air bubbles in the saline

chamber, *J*, when the heart was immersed in it and the chamber sealed. In addition to the adjustments in the pressure and volume of the ventricle made by varying the level of the saline reservoir, *E*, adjustments were possible also by means of a movable 5 cc. pipette, *L*, connected with the saline chamber, *J*. This pipette, *L*, was calibrated to 0.1 cc. and mounted alongside of a millimeter scale, *M*. Both the volume and pressure scale were found to be sensitive enough when large hearts were used.

When recording isometric contractions, the initial pressure could be varied within wide limits without altering the initial volume, simply by changing the level of the pipette, *L*, between records. The same thing could be accomplished by connecting the ventricle for a short interval of time between records to the saline reservoir, *E*, which had been shifted in level, and restoring the volume of the ventricle to its original value by adjusting pipette *L*, and then sealing the perfusion cannula, *I*, by turning stopcock *B*. The procedure was the same when recording auxotonic contractions except that stopcock *B* was always open and stopcock *A* was turned for a short interval between records so that the vertical tube, *G*, instead of the ventricle, was connected with the saline reservoir, *E*.

The only difference in adjustment necessary in the last two methods to change the initial volume and keep the initial pressure constant is in the final adjustment of the pipette, *L*. This adjustment was made to keep the fluid level in the pipette at a constant height of the scale, *M*. Unlike the previous methods this adjustment was made, in isometric contractions, after the perfusion cannula was sealed. The constancy of the initial pressure was checked in the vertical tube, *G*, against the marker, *H*, in auxotonic contractions; and in all cases in the photographic records.

Parallel changes in initial pressure and volume were induced by varying the height of the saline reservoir, *E*, between records and connecting the ventricles temporarily with it. Opposite changes in initial pressure and volume were produced by varying the height of the pipette, *L*, between records and connecting the heart with the vertical tube, *G*, temporarily when recording isometric contractions or permanently when recording auxotonic contractions.

The manometer was calibrated at the end of each experiment so that the pressure could be read in millimeters of saline. No attempt was made in most cases to determine the volume changes other than in relative terms. The notations $+$, $++$ or $-$, $--$ etc. being used to designate the changes. A $+$ or $-$ sign has a value of approximately 0.5 cc. The area bounded by the pressure curve was measured in a few cases. The changes are so clearly visible from the published records however that the data are not included in this report.

RESULTS. The reproductions, of a few typical experiments, used in this report show clearly without much analysis the results obtained. The

changes noted under the four combinations of initial pressure and volume changes were uniform regardless of whether the contraction was auxotonic or isometric. Changes in the rate of the ventricle were rather infrequent. In most of the experiments the ventricular rate was fortunately surprisingly constant during the course of a series of observations. This was true of the ventricular rate in the series reproduced in figures 2 to 7.

In figure 2 is shown the effect when the initial pressure and volume were changed in the same direction. The increase in amplitude, the prolongation in the duration of the curve, and the augmentation of the area bounded by the curve (tension-time) which accompany the elevation in inital pressure and volume are clearly visible. This confirms the observations of Frank (1895), Kozawa (1915), Daly (1923), and Segall and Anrep (1926). Such experiments were made merely as a check to show that the responses of the ventricle were not bizarre.

That these changes in the ventricular contraction are caused by variations in initial volume is shown in the experiments where the initial volume was changed and the initial pressure kept constant. Two such cases are shown in figures 3 and 4. The former illustrates the effect in an auxotonically contracting ventricle and the latter in an isometrically contracting one. In both the initial volume was at first decreased and then increased in one or several stages to a value higher than at the start. The figures show how the amplitude, duration, and tension-time of the pressure curve vary in the same direction as the initial volume in spite of the constancy of the initial pressure.

The dependence of cardiac contraction on initial volume is also shown in experiments in which the initial volume and pressure were varied in opposite directions. The effect in an auxotonically contracting heart is shown in the segments of figure 5. In this case the initial volume was first increased, then decreased in two stages beyond the control value, and then again increased above the control. The changes in initial pressure were just the reverse. Despite the opposite changes in initial pressure, the amplitude, duration, and tension-time value of the pressure curve vary in the same direction as the initial volume. These experiments demonstrate that initial volume regulates the activity of the heart.

It might be argued, however, that this does not rule out the possibility that initial tension still has a definite regulatory influence which is overshadowed by the effect of initial volume changes. This possibility was tested by keeping the initial volume constant and varying only the initial pressure. The usual effect is shown in figure 6. The initial pressure in this experiment was raised to a relatively high level at first, dropped below zero, raised again, dropped by stages, and in the final segment elevated again. The only change in the pressure curve accompanying the wide variations in initial pressure is a very slight progressive decrease in amplitude and tension-

time value, probably the result of fatigue associated with the cessation of perfusion during the course of the series of records, a period of less than 5 minutes. Occasionally, however, as shown in figure 7, the changes in initial pressure are associated with parallel insignificant changes in amplitude and tension-time value, in no way comparable to those accompanying initial volume changes of equivalent range. These tiny variations occurred when the initial pressure was suddenly altered a few hundred millimeters of saline, a change practically outside the physiological range. This last phenomenon probably belongs in the same category as that noted by Edwards and Cattell (1928) with compressions of several atmospheres.

DISCUSSION. The results of the present research indicate clearly that changes in the initial pressure comparable to those encountered in physiological conditions have no effect on the contraction of the ventricle, or at most only an insignificant one. Apparently the pressure to which the surface of the ventricle is exposed, which acts as lateral pressure and by compressing the ventricular substance and increasing its tension, is without

Fig. 2. Three pressure curves of an auxotonically contracting ventricle recorded in an experiment in which the initial pressure and volume were both increased progressively between records (reduced ¼). The initial volume in the second segment was increased ++++ above the first, and in the third ++ above the second (+ is equivalent approximately to 0.5 cc.). In this and succeeding figures the black horizontal line near the top is the base line. The pressure readings can be made from the scale on the left whose ordinate values are in millimeters of saline. They should be made from the base line. Time in seconds is indicated by notching of white line at bottom of records. A hand lens will bring out the details more clearly. Discussed in text.

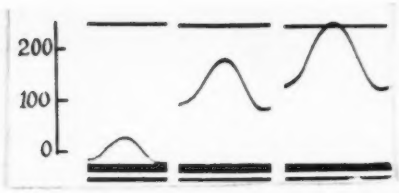
Fig. 3. Three pressure curves of an auxotonically contracting ventricle recorded in an experiment in which the initial volume was varied while the initial pressure was kept constant (reduced ¼). Initial volume decreased −−− between first two segments and increased ++++++ between last two. Time and scale as in figure 2. Discussed in test.

Fig. 4. Five pressure curves of an isometrically contracting ventricle recorded in an experiment as in figure 3 (reduced ¼). The initial volume was decreased −−−−− between the first and second segment, increased ++++, + and + respectively between the second and fifth segments. Time, base line and scale as in figure 2. Discussed in text.

Fig. 5. Five pressure curves of an auxotonically contracting ventricle recorded in an experiment in which the initial volume and pressure were varied in opposite directions (reduced ¼). Initial volume increased + between first two segments, decreased −−−− and − between the second and third, and third and fourth segments respectively, and then increased ++++++ between the last two segments. Time, base line and scale as in figure 2. Discussed in text.

Fig. 6. Seven pressure curves of an isometrically contracting ventricle recorded in an experiment in which the initial volume was kept constant and the initial pressure varied (reduced ¼). Time, base line and scale as in figure 2. Discussed in text.

Fig. 7. Six pressure curves of an isometrically contracting ventricle recorded as in figure 6 (reduced ¼). Time, base line and scale as in figure 2. Discussed in text.



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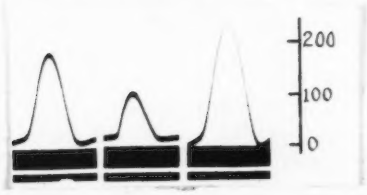

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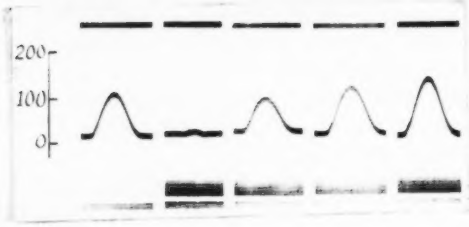

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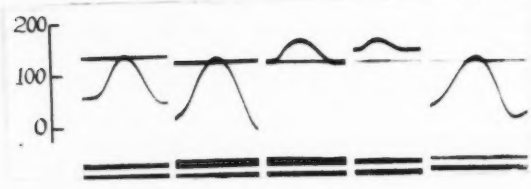

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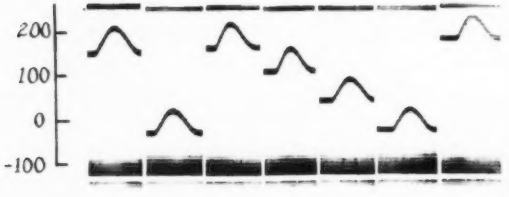

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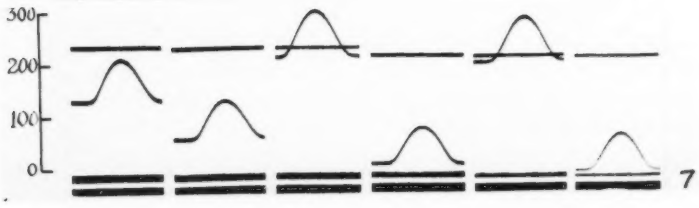

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influence on cardiac activity. On the other hand, the initial volume of the ventricle exerts a profound effect on its contraction regardless of the nature of the accompanying changes in initial pressure. This effect is not only on the amplitude and duration of the pressure curve but, as was already pointed out, on the tension-time value as well. Inasmuch as the tension-time value is an index of the mechanical energy[1] set free when the contraction is isometric (Hartree and Hill, 1921), the changes in tension-time values observed in isometric contractions such as figure 4 show that initial volume operates by changing the mechanical energy which the heart muscle liberates.

How does the change in initial volume produce its effect? This subject has been discussed at great length, both for isometric and auxotonic contractions by Gesell (1916, 1920). At present, the discussion will be limited to a few of the more important factors accompanying the increase in initial volume which might modify the magnitude of an isometric contraction. The pressure developed in an isometric contraction is a measure of the tangential force developed by the ventricle per unit area of its internal surface. As the initial volume increases the muscle fibres are spread over a greater area and consequently their effectiveness is decreased (cf. Klein, 1896; Gesell, 1916). This factor tends to counterbalance the mechanism which augments cardiac activity. On the other hand, as the volume increases the individual fibres of the heart are straightened and placed more nearly in the tangential plane. Inasmuch as it is the tangential fibres which exert the greatest force, this factor tends to increase the total force of the heart but hardly enough to account for the changes observed. Some physiological mechanism must therefore be involved. Blix (1895, 1902)and later A. V. Hill (1913) attributed the augmentation in force to the greater energy exchange which results from an increase in the surface area of the fibres where the chemical changes occur. It is possible, however, that some other variable accompanying the stretching of the muscle is responsible for the greater energy released. In fact, the present research does not rule out the possibility that tension changes in the muscle resulting from the lengthening regulate the response, provided one assumes that the effect of the change in tension produced by lengthening is different from that following the tension changes associated with the variations in hydrostatic pressure. This possibility the author believes to be extremely unlikely. The solution of the entire mechanism is at present still a matter of speculation. But, even though the present research does not help to settle how initial volume changes operate, it shows beyond question that the regulation of cardiac activity is by the initial length of the ventricular muscle,

[1] In reality it is only an index of the intensity factor of the mechanical energy. It becomes an index of the mechanical energy when the changes in the capacity factor (length) are in the same direction, as in these experiments.

regardless of how it is produced; and furthermore, that changes in initial intraventricular pressure in the physiological range exert practically no effect per se.

SUMMARY

1. A method is described by which it is possible to dissociate initial pressure and volume while keeping the rate and other conditions of the isolated turtle ventricle constant.

2. The effect of such dissociations on the pressure developed in isometrically and so-called auxotonically contracting ventricles was determined.

3. A method is described for recording the pressure variation of the turtle's ventricle with a sensitive optically recording manometer of the Wiggers pattern.

4. It was found that the amplitude, duration and the area bounded by the pressure curve increased with an increase in initial volume regardless of whether the initial pressure was kept constant or changed in the same or opposite direction. These observations establish the fact that it is initial volume which regulates the activity of the ventricle when the distention of the heart changes.

5. Changes in initial pressure amounting sometimes to two hundred millimeters of saline were usually without effect on the amplitude, duration and tension-time value of the pressure curve provided the initial volume was constant. Occasionally, slight changes in amplitude and in tension-time value in no way comparable to those produced by equivalent changes in initial volume, were observed. These tiny variations occurred when the initial pressure was changed suddenly several centimeters of saline, changes practically outside the physiological range. These observations show that initial pressure changes per se, comparable to those encountered in physiological conditions, have no effect on the contraction of the heart or, at most, only an insignificant one.

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FURTHER OBSERVATIONS ON DECREMENT IN NERVE CONDUCTION[1]

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The unsettled question of the existence of decrement in nerve conduction continues to be of great interest in relation to our understanding of the nature of the nerve impulse. The earlier experimental work has been reviewed by Kato (1924, 1926) and by Davis (1926) and will not be covered here. The older conception that there is a continuous decline in the intensity of the nerve impulse in passing through a region rendered uniformly unfavorable to conduction through the action of such agencies as narcotics or pressure, has largely given way to the view that the impulse travels with a uniform but reduced intensity through such regions, i.e., it is determined solely by the local condition of the nerve. The suggestion of Davis, Forbes, Brunswick and Hopkins (1926), based on theoretical considerations, that the impulse does not fall immediately to the lower level characteristic of narcosis but is first conducted with a decrement for a short distance, has received support in investigations from this laboratory. It was found (Cattell and Edwards, 1927) that when the nerve was compressed with hard rubber blocks the time required to stop conduction under a given pressure per unit length of nerve was the same for 8 mm. and 16 mm. distances, but when the pressure involved a length of 4 mm. it took nearly twice as long to block conduction in a majority of the fibers. This result appears to indicate a transitional decrement in intensity of conduction extending between 4 and 8 mm. into the compressed region. More recently Shen, Hou and Lim (1927) have studied the effect of cooled areas on the rate of blocking conduction in the phrenic and vagus nerves of the dog, and they conclude that conduction with decrement takes place over the first 5 mm. of cooled nerve but without decrement over longer lengths. Their results, however, are inconclusive and may be due, as they suggest, to a more rapid and complete cooling in those nerves exposed to a greater cooling surface, and therefore may not represent a true decrement.

Our earlier experiments are open to the possible objection that the results might be modified in some way by the method employed of compression

[1] A preliminary summary of this work appeared in the proceedings of the American Physiological Society, This Journal, 1927, lxxxi, 472.

between hard rubber surfaces. Pressure might not be transmitted equally to all the fibers in the nerve trunk, and there would necessarily be deformation of the nerve and possibly greater injury at the boundaries of the compressed region by the edges of the rubber blocks. It appears to us, however, that these factors would operate similarly in all experiments, and therefore would not invalidate comparison of the speed of blocking in different compression distances. Nevertheless, it seemed desirable to check our earlier work, using an entirely different method for the application of pressure. This we have done by the use of a device for the trans-

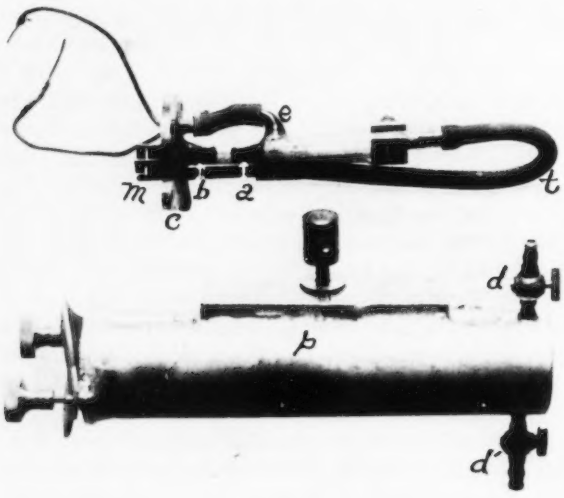

Fig. 1. Apparatus used for applying pressure through a liquid system to localized areas of nerve. Explanation in text.

mission of pressure to the nerve through a fluid medium, and the results of these experiments form the basis of the present paper.

METHOD. The form of the apparatus used is illustrated in figure 1. It consists essentially of two glass cannulae, *a* and *b*, mounted on the head of the pressure cylinder, *c*. Attached to the tips of these cannulae and extending the distance between them is a segment of a dog's artery or vein (not shown in illustration) through which the nerve is passed. Pressure is applied externally through the liquid medium contained in the cylinder, *p*, and it acts on a length of nerve determined by the separation of the two cannulae. The adjacent regions of the nerve are maintained at

atmospheric pressure through connections to the exterior from opposite ends of the cannulae. One cannula, *b*, through which the nerve enters, is attached directly to the cylinder head, while the other, *a*, connects with the exterior through a short length of non-collapsible tubing, *t*. Connections from a pair of platinum electrodes leave this cannula through a side arm, *e*, passing to the exterior through a short length of brass tubing soldered into the cylinder head.

As in the earlier series of experiments most of the observations have been made on the sciatic nerve of the frog, using the response of the attached gastrocnemius muscle as an indication of the number of nerve fibers conducting. The nerve-muscle preparation was isolated from the animal except the muscle origin near the knee joint which was left intact. A pin passing through this joint was inserted into the muscle support, *m*, on the head of the cylinder. The tendinous end was attached to a torsion wire isometric lever, through which tension changes were recorded on smoked paper. In order to keep a constant initial tension of about 50 grams on the muscle, the pressure chamber including the muscle clamp was mounted on an adjustable clamp so arranged that the distance between it and the lever could be altered by turning a thumb screw. The nerve from the muscle was threaded through the two cannulae and intervening segment of blood vessel and the distant end laid across the stimulating electrodes within the second cannulae.

The head, *c*, with its attachments is inserted into place on the pressure chamber, *p*, after mounting the preparation and is tightly screwed in place to prevent leakage. Through connections, *d*, *d'*, on the top and bottom the chamber is then filled with Ringer's solution. Pressure applied to this fluid is transmitted through the blood vessel to the nerve, i.e.. to the part unprotected by the glass cannulae situated between the electrodes and the attached muscle. Pressure is applied to the chamber from a tank of compressed oxygen. A second chamber, partly filled with the solution, is interposed betweeen the chamber containing the nerve and the source of pressure for convenience in filling the first chamber and also to avoid possible effects from a change in the concentration of dissolved oxygen. A mercury manometer is connected with the chamber to indicate the degree of pressure which in these experiments was uniformly applied at a pressure of 1250 mm. of Hg. The method just described utilizes the principles employed by Meek and Leaper (1911). In the form used in our experiments it presents the advantage of giving a sharply localized area of pressure surrounding the nerve trunk without causing stretching. Moreover, all difficulty from leakage is completely eliminated.

The carotid artery of a medium sized dog was found to be the most satisfactory for transmission of the hydrostatic pressure to the nerve. Strips of vessel can be placed in glycerol for preservation. Prior to use a small segment is placed in Ringer's solution for a short period until softened.

In the present series of experiments measurements were made of the time required to suspend conduction when the pressure was applied over an 8 mm. length of nerve and when applied over 4 mm. If the intensity of the changes accompanying the nerve impulses is uniformly depressed in the compressed region then the period required to block conduction should be the same regardless of the length of nerve involved. On the other hand if the impulse travels with a decrement through the compressed region, the time of extinction would be directly related to the length of nerve involved. A transitional decrement extending over 4 mm. into the compressed part would result in a more rapid blocking of the impulse in an eight millimeter compression distance than in four millimeters.

The general procedure in carrying out an experiment was similar to that described in our previous paper. Control tension records following the application of a short tetanic stimulus were made before pressure was applied and at intervals of one minute while it was acting. The pressure used (1250 mm. of Hg) was continued until the muscle response practically ceased, usually after a period of between eight and fifteen minutes. At the end of the experiment, in order to test the condition of the muscle, a record was made of the tension developed when the stimulus was applied to the nerve between the muscle and the point of blocking. The changes in the maximum tension developed during the application of pressure were used as an approximate indication of the relative number of fibers still functioning at any instant.

Results. The experiments were carried out in two series: In the first observations were obtained on a number of preparations in which the nerve was subjected to pressure over a distance of 4 mm. and later observations for the 8 mm. distance were made. The results from this group of experiments are tabulated in tables 1 and 2. The figures show the time required for the pressure to block 10 per cent, 50 per cent, and 80 per cent of the fibers as indicated by the falling off of the tension developed by the muscle. The percentages given were arbitrarily selected and the figures for time were obtained by measurement and interpolation of the kymograph records. There is a relatively large error in the measurement of the initial and final changes in tension and it is for this reason that the intermediate changes only have been tabulated.

There is a significant difference in the time required to suspend conduction in the two compression distances studied. The average time required to produce a given tension change is approximately 50 per cent longer in those experiments in which the pressure acted on the shorter length of nerve. This method of applying pressure thus gives results essentially the same as those previously reported for the effects of block compression.

It is apparent from inspection of the tables that the individual variations are considerably smaller between observations made on the two prepara-

tions from the same animal than they are between those taken at random. We have not been able to discover the basis for the varying susceptibility

TABLE 1

The effect of a pressure of 125 cm. of mercury applied over an 8 mm. length of nerve

The figures in this and subsequent tables represent the time in minutes required for the reduction of the tension developed by the muscle to the extent indicated at the head of the column.

EXPERIMENT	10 PER CENT REDUCTION			50 PER CENT REDUCTION			80 PER CENT REDUCTION		
	Left	Right	Difference	Left	Right	Difference	Left	Right	Difference
	minutes	*minutes*	*minutes*	*minutes*	*minutes*	*minutes*	*minutes*	*minutes*	*minutes*
4	2.4	2.6	+0.2	3.8	3.9	+0.1	5.9	5.1	−0.8
5	1.4	1.6	+0.2	3.3	3.9	+0.6	6.4	6.8	+0.4
6	1.1	1.7	+0.6	2.9	3.4	+0.5	5.2	6.0	+0.8
14	1.6	1.4	−0.2	2.8	2.9	+0.1	3.8	3.8	0
15	1.3	1.9	+0.6	3.4	3.6	+0.2	4.9	5.7	+0.6
16	3.1	2.3	−0.8	9.9	6.1	−3.8	13.2	9.8	−3.4
17	4.1	3.1	−1.0	5.6	5.5	−0.1	6.7	7.5	+0.8
Averages	2.14	2.09	±0.51	4.53	4.19	±0.77	6.59	6.39	±0.97
	2.11			4.36			6.49		

TABLE 2

The effect of a pressure of 125 cm. of mercury applied over a distance of 4 mm.

EXPERIMENT	10 PER CENT REDUCTION			50 PER CENT REDUCTION			80 PER CENT REDUCTION		
	Left	Right	Difference	Left	Right	Difference	Left	Right	Difference
	minutes	*minutes*	*minutes*	*minutes*	*minutes*	*minutes*	*minutes*	*minutes*	*minutes*
1	3.6	3.2	−0.4	6.7	6.8	+0.1	8.7	11.3	+2.6
2	3.8	4.3	+0.5	7.0	7.5	+0.5	10.3	11.5	+1.2
3	7.2	3.4	−3.8	11.2	12.3	+1.1	16.0	20.0	+4.0
7	3.3	2.5	−0.8	6.2	4.7	−1.5	9.7	8.4	−1.3
8	2.0	1.5	−0.5	4.4	4.7	+0.3	8.1	9.3	+1.2
9	4.2	3.7	−0.5	12.3	10.0	−2.3	18.0	16.6	−1.4
10	4.2	3.4	−0.8	6.3	5.6	−0.7	9.5	9.1	−0.4
11	2.2	1.3	−0.9	3.5	3.6	+0.1	5.8	5.6	−0.2
12	2.0	1.9	−0.1	3.5	3.0	−0.5	4.8	3.8	−1.0
13	2.3	2.0	−0.3	4.4	3.9	−0.5	7.1	6.5	−0.6
Averages	3.48	2.72	±0.86	6.55	6.21	±0.76	9.80	10.21	±1.39
	3.10			6.38			10.00		

of the nerves of different animals to pressure, but it has been observed repeatedly both in our earlier experiments and those reported here. In one instance on using frogs from a new shipment the given pressure acted

more than twice as rapidly as it had on those previously used. Because of this variability a second group of experiments has been carried out in which the four and eight millimeter compression distances have been applied respectively to the two preparations from the same animal. The results are summarized in table 3. Except for the initial changes, i.e., the blocking of conduction in the first few fibers to be affected, they uniformly show more prolonged conduction in the case of the shorter compression distance. The average differences agree well with those found in the first series, but since the variability has been reduced by the method of obtaining figures for both compression distances from the same animal, the results have a greater significance. This series of experiments is also

TABLE 3

The influence of compression distance on the time required to suspend conduction in preparations of the sciatic nerve taken from the same animal

EXPERIMENT	10 PER CENT REDUCTION		50 PER CENT REDUCTION		80 PER CENT REDUCTION	
	4 Mm.	8 Mm.	4 Mm.	8 Mm.	4 Mm.	8 Mm.
	minutes	*minutes*	*minutes*	*minutes*	*minutes*	*minutes*
1	2.1	2.8	7.2	5.2	11.8	8.0
2	1.7	1.9	5.4	3.4	11.4	7.0
3	2.4	1.5	6.2	3.3	13.0	6.0
4	2.5	2.2	6.4	5.9	13.7	7.5
5	2.1	1.5	4.2	3.1	9.8	4.8
6	1.4	2.3	3.3	3.6	9.6	7.4
7	2.1	2.2	4.9	4.2	10.3	5.5
8	1.5	1.8	4.5	4.4	11.6	6.7
9	1.5	1.3	6.3	3.2	15.1	6.8
10	1.1	1.1	2.8	2.9	6.0	4.8
11	2.1	2.3	4.2	4.6	6.2	6.2
12	1.7	1.0	4.1	2.6	8.7	3.6
Averages	1.85	1.82	4.95	3.86	10.60	6.19

represented in the chart reproduced as figure 2, in which each point represents the average of the twelve experiments. Figures have been calculated by interpolation of the original tension records of the time required to stop conduction in from 10 to 80 per cent of the fibers at intervals of 10 per cent changes. The upper curve shows the tension changes resulting from compression applied over a 4 mm. length of the nerve, while the lower curve gives the results for the 8 mm. compression distance. The divergence of these two curves shows in a striking manner the more rapid blocking of conduction in the longer of the two compression distances as the experiment is continued. On the other hand the initial changes, i.e., the blocking of the first twenty per cent of fibers is quite independent of the compression distance involved.

DISCUSSION. The fact that a majority of nerve fibers cease to conduct sooner when pressure is applied over a distance of 8 mm. than they do when a similar pressure is applied over 4 mm. leads to the conclusion that the nerve is conducting with a decrement through the unfavorable region. In our previous experiments (Cattell and Edwards, 1927) with block compression we found no difference in the influence of pressure applied over 8 mm. and 16 mm., thus confirming earlier work by Kato (1924) and by Davis, Forbes, Brunswick and Hopkins (1926) showing that the nerve conducts without decrement over distances greater than eight millimeters. Further experiments, therefore, have not been carried out with compres-

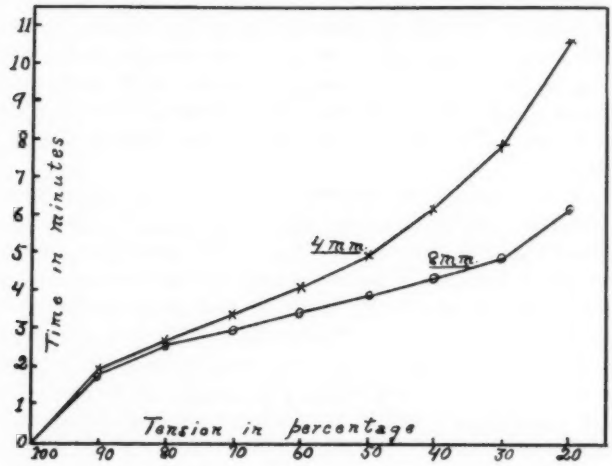

Fig. 2. Graph showing the greater effectiveness in blocking conduction of an 8 mm. compression area as compared with a 4 mm. area in a series of twelve experiments. Details are given in the text.

sion distances greater than eight millimeters, beyond which distance we may conclude that the nerve impulse has reached an equilibrium, its intensity now depending only upon the local condition of the nerve. The present experiments confirm the earlier ones in showing a transitional decrement in the intensity of the conduction of the nerve impulse extending more than four millimeters into the compressed region.

From the curves shown in figure 2 it is apparent that in this series of experiments the initial change occurs at about the same time in both compression distances, thus contrasting with the effect of the pressure on a majority of the fibers. While this has not been uniformly observed it also occurred in the experiments in which the nerve was compressed between

hard rubber surfaces and it undoubtedly is of significance. It must obviously be due to some factor other than the length of nerve compressed which determines the time that a majority of the fibers cease to conduct. In our earlier report we gave reasons for believing that this initial blocking was due to an action of the edges of the compression blocks in injuring those fibers which were nearest to the block. We hoped to avoid this factor in the present experiments in which pressure was applied through a fluid medium, but as they proceeded it became apparent that this difficulty is inherent in the pressure method of blocking conduction.

The influence of pressure in suspending functional activity is dependent upon a difference in pressure between two points in the tissue which results in a displacement of material and deformation. Within limits pressure *per se* has no depressant action on tissue function as we have shown by subjecting cardiac tissue (Edwards and Cattell, 1928) and nerve muscle preparations (Cattell and Edwards, 1928) to pressures up to 1000 pounds per square inch. The only effects observed have been in the direction of stimulation.

Since, in the experiments described in the present paper, pressure has been applied to a sharply localized length of nerve it is to be expected that its influence would be the most marked at the boundaries, beyond which the hydrostatic pressure is balanced only by the atmospheric pressure. That this is the case was clearly shown by examining the nerves following compression in the chamber. They invariably showed two areas of constriction marking the limits of the area acted upon by the pressure, while the intervening area appeared practically normal. Such nerves have been examined histologically and it has been found that at the limits of the compressed region there is greater compression of the myelin sheath and that the clear area surrounding the axis cylinder is much reduced, indicating a greater fluid displacement in this region. There seems no reason to assume nor have we evidence that this factor is more important in the longer of the two compression distances studied. If anything the reverse might be true, for there are some grounds for expecting a greater displacement of material when pressure acts over a shorter distance.

The deforming action of pressure occurring at the limits of the compressed region gives an adequate basis for the explanation of the initial action in blocking approximately twenty per cent of the motor fibers of a nerve trunk, which may occur in the same period of time regardless of the area compressed. The more gradual influence of pressure extending over the whole length of nerve compressed is unquestionably the factor which determines the variation in the time of blocking with the length of the compressed region. In other words, with all other conditions constant we find that a majority of the fibers (i.e., those which retain their function the longest) continue to conduct longer when the compressed region is

less. This result confirms the earlier work with block compression and indicates conduction with a decrement extending at least four millimeters into the unfavorable area.

SUMMARY AND CONCLUSIONS

1. A method is described for the application of sharply localized hydrostatic pressure to tissues.

2. The influence of pressure in blocking conduction is entirely dependent upon deformation of the tissue due to its unbalanced action.

3. The initial effect of pressure in stopping conduction in the frog's nerve is probably due to the marked displacement of material occurring at the boundaries of the compressed region and its speed of action is independent of the distance compressed.

4. The survival period of the majority of fibers (i.e., those escaping the initial action of the pressure) varies with the area compressed. On the average it took approximately fifty per cent longer to block conduction when the pressure acted over a 4 mm. length of nerve than it did for 8 mm.

5. These results confirm previous work and indicate that the nerve impulse travels with a decrement at least 4 mm. into the compressed portion of the nerve.

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THE SHERRINGTON PHENOMENON

I. The Nerve Fibers Involved in the Sensitization of the Muscle
II. The Nerve Fibers Which Produce the Contraction
III. Antagonism by Adrenalin

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I. After section and degeneration of the hypoglossal nerve on one side, it was shown by Vulpian and Philippeaux (1) that contraction of the corresponding half of the tongue was produced by stimulation of the chorda lingual nerve or the chorda tympani nerve alone. This was confirmed by Heidenhain (2) who called it a pseudomotor response. Sherrington (3) observed a reaction in the denervated mammalian limb muscles which is similar to the pseudomotor reaction. He sectioned the 6–7 L and the 1–2 S dorsal and ventral roots in the cat between the spinal ganglia and the spinal cord. After 42 days, the application of strong induction shocks to the peripheral end of the cut sciatic nerve caused contraction of the muscles controlling the ankle joint and the foot. Sherrington was tempted to associate it with contraction of the muscle spindles due to stimulation of their sensory fibers. He ruled out the possibility that the contraction might be caused by a spread of the stimulus to the muscle for he found that the reaction occurred when the stimulation electrodes were 20 cm. from the muscle.

Van Rijnberk (4) was able to repeat Sherrington's results. Upon section of both ventral and dorsal roots between the dorsal root ganglia and the spinal cord, he found that, after sufficient degeneration time, stimulation of the sciatic nerve caused contraction of the muscles of the lower leg and foot. When he sectioned only the ventral roots and left the dorsal roots intact with the spinal cord, he was unable to produce the phenomenon. He suggested that, in order for it to appear, fibers coursing in the dorsal roots with cells of origin within the cord must be cut and degenerated. He presented very important evidence to show that the thoracolumbar sympathetic fibers were not the ones carrying impulses responsible for this phenomenon. At a time when a response could be obtained by stimulation of the sciatic nerve, he was unable to evoke a

reaction by stimulation of the thoracolumbar sympathetic trunk alone. Furthermore, a response was obtained in a nerve in which the postganglionic thoracolumbar sympathetic fibers as well as the ventral root fibers had been degenerated and only fibers coursing in the dorsal roots were present. Sherrington (3) excised the spinal ganglia and sectioned the ventral roots of the nerves supplying the sciatic. The postganglionic sympathetic supply would have been intact in the sciatic nerve of these preparations but the phenomenon failed to appear. The evidence available shows that this abnormal type of response is called forth by impulses travelling over fibers which have their cells of origin in the dorsal root ganglia and would belong to what we recognize as sensory fibers.

Heidenhain found that the denervated tongue was caused to contract when nicotine was injected intravenously. Frank, Nothmann and Hirsch-Kaufmann (5) observed that such a contraction as well as that of denervated limb could be produced by acetyl-choline, and Dale and Gasser (6) determined that the action was one held in common by drugs having a nicotine-like action, such as the quaternary bases and cytisine.

If Van Rijnberk be correct in his observation of the absence of the Sherrington phenomenon when the dorsal roots are left intact and only the ventral roots degenerated, one would also expect a failure to obtain a contracture with acetyl-choline under these conditions. This deduction is based on the fact that the contractures of denervated muscle produced by this group of drugs seem to be associated with the appearance of a slow contraction on stimulation of certain other nerve fibers, in the tongue phenomenon of fibers in the chorda tympani nerve, and in the Sherrington phenomenon of fibers which have their cells of origin in the dorsal root ganglia. Whatever takes place in the muscles to make possible these phenomena should also sensitize them to drugs, and, therefore, tests with the latter may be added to the direct determination of the results of stimulation of the residual fibers.

With the above considerations in mind experiments were undertaken to ascertain whether the presence of intact dorsal roots would completely prevent the drug contracture and the Sherrington phenomenon.

Method. Intradural section of the ventral roots of the lumbosacral plexus was performed in adult cats according to the technique described by Ranson (7). After 10 to 16 days, the cats were examined for the reactions in which we were interested. The tensions developed in the denervated gastrocnemii were recorded by using the steel spring tension lever with the method of Dale and Gasser (6). The Sherrington phenomenon was obtained by applying strong rapidly alternating induction shocks from a Harvard coil to the peripheral end of the cut sciatic nerve in which the ventral root component alone had been degenerated. Drug contractures were produced by intra-arterial injection of 1 cc. of a 1:1000

solution of acetyl-choline into the internal iliac artery on one side. The arterial branches were so clamped off that the drug would be delivered to one limb or the other as desired. The muscle fibers were stimulated directly to produce maximal tetanic contractions by applying rapidly alternating induction shocks through silver wire electrodes coated with silver chloride. Two such electrodes were used, one piercing the belly of the muscle and the other transfixing the Achilles tendon.

Our operative procedures were controlled by autopsy and histological preparations. Marchi preparations of the spinal cord and the spinal roots were examined. Sections of the cord showed scattered degeneration, varying in amount. This degeneration was due probably to anemia and to the adhesion of the cicatrix to the cord. It was found scattered in all funiculi of the cord and in the central gray matter.

Results. In our first series of four cats, the 4–5–6–7 L and 1–2 S ventral roots on the right side were cut intradurally and two weeks were allowed for somatic motor degeneration. These cats were operated for another purpose and were decerebrated by the transection method. All four cats showed positive drug contractures of the gastrocnemii in the presence of intact dorsal roots.

In the second series of 6 cats, the right 6–7 L and 1–2 S ventral roots were cut and the dorsal roots of these respective nerves were left joined with the spinal cord. The left sciatic nerve was cut in the thigh in each cat. Provided sufficient degeneration time is allowed, this procedure should result in the partial denervation of the right gastrocnemius, the dorsal root component being intact, and complete denervation of the left gastrocnemius. To be sure, the sciatic nerve on the right side would contain post-ganglionic thoracolumbar fibers. However, Van Rijnberk has shown that these fibers do not carry the impulses responsible for the Sherrington phenomenon.

After 10 to 16 days, the cats were put under ether and the reactions of the two sides compared. The Sherrington phenomenon was found to be present in every one of the six cats on stimulation of the sciatic nerve and was very definite as is shown in figure 1. The question now arises as to whether the reaction could be due to the few degenerated fibers found in the dorsal root and cord. It receives a negative answer as the result of a number of physiological considerations. The limb was exquisitely sensitive to stimulation; and that the muscles themselves had their sensory innervation functionally intact with the central nervous system was shown by the fact that manipulation of the right gastrocnemius caused reflex responses in other parts of the body. Furthermore, it would seem that Sherrington phenomenon contractions with the tensions shown in table 1 could not be attributed to the presence of scattered degeneration. But the degree of sensitization could be put to a more decisive test by com-

paring the tensions produced in the drug contractures on the partially denervated and the completely denervated sides under otherwise the same physiological conditions. This was done by first finding in each case the ratio of the contracture tension to the maximal tension of the muscle resulting from direct tetanic stimulation, and then comparing the ratios. In table 1, we see that these ratios show slight variations in the same animals but the reactions are so nearly alike that we feel justified in saying that they are symmetrical on the two sides. It is evident that drug contractures not only appear in skeletal muscle in which the dorsal root component is left intact with the spinal cord but that they are equal in magnitude to those in skeletal muscle which is completely denervated.

Figure 1 shows a record from a typical experiment. In figure 2 the drug contracture is greater than in the other experiments. Here the in-

TABLE 1

In these cats the right 6–7 L and 1–2 S ventral roots and the left sciatic nerve were cut. Ten to sixteen days' degeneration time was given.

NUMBER	SHERRINGTON PHENOMENON	RIGHT MAXIMAL CONTRACTION	RIGHT DRUG CONTRACTURE	RATIO	LEFT MAXIMAL CONTRACTION	LEFT DRUG CONTRACTURE	RATIO
	kgm.	*kgm.*	*kgm.*	*per cent*	*kgm.*	*kgm.*	*per cent*
P	1.2	1.3	0.9	70	3.3	2.5	76
Q	1.4	4.2	2.1	50	5.5	2.6	47
R	1.4	8.2	2.9	35	8.0	3.4	42
V	0.63	4.7	1.0	21	6.0	1.3	22
W	0.37	5.4	1.4	26	8.2	1.9	23
X*	2.3	9.0	6.2	69			

* In cat X, acetyl choline was injected directly into the femoral artery. The left femoral artery was damaged so that the reading on the left side is not included.

jections was made directly into the femoral artery instead of into the internal iliac and under these conditions the drug undoubtedly reached the muscle in greater concentration. The amount of tension evidently depends on the concentration at which the drug reaches the muscle fibers.

Discussion. The evidence as presented here does not support the view that there are fibers with cells of origin in the spinal cord coursing in the dorsal roots which must be cut before the Sherrington phenomenon will appear. The same may be said for the occurrence of drug contracture with acetyl-choline. The occurrence of the reaction of Vulpian depends upon section and degeneration of the hypoglossal nerve on the corresponding side. By this section, fibers responsible for proprioceptive sensation are probably cut and degenerated as was shown by Boeke (8) and by Langworthy (9). To obtain this reaction, fibers coursing in the chorda tympani nerve on the same side must be stimulated. These fibers which

are brought into activity are connected with the brain stem. However, this does not interfere with the reaction nor does it prevent drug contracture. If there be fibers with cells of origin in the brain stem which inhibit this reaction they must course in the hypoglossal nerve.

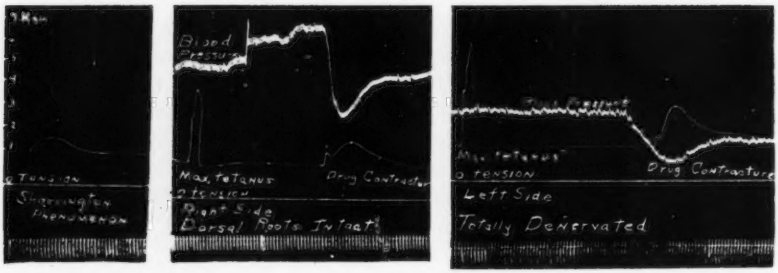

Fig. 1. Cat Q. December 6, 1927. Ventral roots 6–7 L and 1–2 S were cut on right side and the sciatic nerve was cut in the thigh on the left side. December 22, 1927, the experiment was performed; 1 cc. of 1:1000 acetyl-choline was injected into the left internal iliac to produce drug contractures. Time, 2 second intervals.

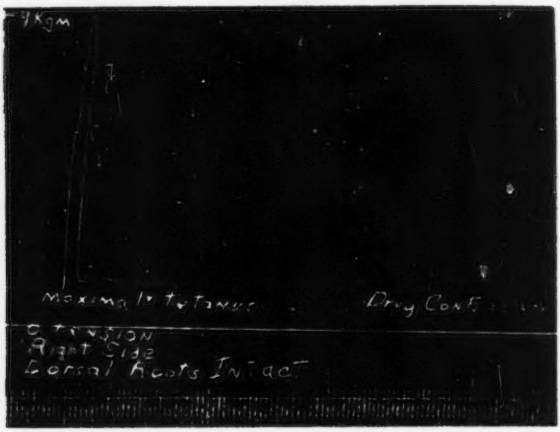

Fig. 2. Cat X. December 26, 1927. Ventral roots 6–7 L and 1–2 S were cut on right side and the sciatic nerve was cut in the thigh on the left side. January 11, 1928, the experiment was performed; 1 cc. of 1:1000 acetyl-choline was injected directly into the femoral artery on the right side. Time, 2 second intervals.

The change in the muscle which takes place before the Sherrington phenomenon and drug contracture appear depends upon section of fibers in the ventral roots. There are two types of fibers to be taken into consideration, somatic motor fibers with cells of origin in the anterior horn and

preganglionic sympathetic efferent fibers with cells of origin in the intermediolateral cell column. However, in the spinal nerves where the ventral roots have been cut in this work, the preganglionic sympathetic fibers are not present. Thus it would seem that section and degeneration of the somatic motor fibers alone is required to sensitize skeletal muscle to the Sherrington phenomenon and to drug contracture.

When the muscle is completely denervated, the contracture appears with acetyl-choline. Likewise it occurs when the dorsal root component and thoracolumbar sympathetic supply are functionally connected with the central nervous system after the somatic motor fibers are degenerated. Thus the presence or absence of the thoracolumbar sympathetic fibers is of no apparent significance in acetyl-choline contracture.

II. The nerve fibers which produce the contraction. When the motor fibers in the nerve innervating a skeletal muscle have degenerated it can be made to contract by stimulation of the dorsal root component of the nerve, but it is not known what fibers in the afferent group are concerned. It has been the general experience of all workers on this subject that very strong repeated stimuli are necessary and this fact suggests that fibers of low irritability are involved, presumably small fibers. That the fibers are smaller than the average follows from the observation that the tongue phenomenon is elicited by stimulation of the chorda tympani; but even this nerve contains fibers of all sizes up to 8μ. A more exact method of localization of the significant fibers is available in the action potential wave and the following experiments were designed making use of it.

Method. The roots were cut on one side in the same segments as before, both dorsal and ventral roots in this case being divided on account of the greater ease in operation. After time had been allowed for degeneration, the preparation was mounted as indicated in Part I with the difference that a weaker spring was used in the tension lever to permit a greater excursion, and that the tibial nerve was divided at its point of emergence from the pelvis and dissected free from the peroneal, thus affording a long stretch for study. It was then mounted on electrodes. At the central end were placed a pair of Ag-AgCl electrodes for the purpose of leading off the action potential. One was placed on the end of the nerve, which had been killed by heat, the other on the intact side. The records were made with a cathode ray oscillograph after three panel amplification, the leads being such that the killed end of the nerve was connected to the grid of the first tube and the intact side to the ground. A stimulating electrode was placed between the leads and the muscle, in some experiments half way between them, in others, close to the muscle in order to gain a larger conduction distance between the stimulus and lead.

The theory on which the experiments are based is as follows. As the

stimulus is increased in size the action potential wave grows through the bringing into activity of fibers having progressively higher thresholds of stimulation. In as much as the conduction velocity in these higher threshold fibers is less and the records are made after conduction, activity in these fibers at the leading off electrode will occur later than it does in the more irritable ones. The potential wave will thus be progressively prolonged with each increment of fibers, and if the mechanical response of the muscle be observed at each strength of stimulus, the part of the potential attributable to the effective fibers may be ascertained by subtraction of the potential wave produced by a subminimal stimulus from those produced by minimal and maximal ones. Then since the velocity can be determined from the time elapsing between the stimulation artefact and the difference potential and since the velocity has a close inverse relationship to diameter of the nerve fibers (10) it was hoped that the fibers could be identified. However, since the strengths of stimuli necessary to produce the contracture were so much greater than those ordinarily used in nerve experimentation, unexpected difficulties arose which made the success of the experiment only partial.

Rate and strength of stimulation. The nerve was excited by a Harvard coil with the core in place, the primary current being broken by a rotating interrupter. The stimulating electrodes were connected to the induction coil so that the cathode was toward the leads, when the form of the action potential was observed, and toward the muscle during the contraction tests. The effect of the rate of stimulation was determined in some preliminary observations. Two facts became apparent: 1, that no matter what the rate of stimulation no response in the muscle takes place when the strength of stimulation is below a limiting value; 2, that with an adequate stimulus the size of response depends upon the rate of stimulation. These facts are illustrated in figure 3. In part A, 21 break shocks per second are compared with 53; in part B, the responses to 53 breaks per second are compared when the strengths of stimulation were determined by using coil separations of 10.5, 5.9 and 2.3 cm. The observed relationship can be interpreted to mean that, the rate being constant, increasing the strength brings more nerve fibers into activity, while, the strength being constant, increasing the rate increases the effectiveness of each fiber involved.

When the makes are allowed to stimulate, the rate is not perfectly controlled because, as the strength of stimulation is increased and the make shocks become adequate, the rate of response becomes doubled in an increasing number of fibers. Furthermore, mammalian nerve, unless it is very fresh, does not give full sized responses at room temperature even when the latter is as high as 26°C. and the rate of stimulation is as low as fifty per second. The size of response then varies with the frequency of stimulation. The result of this is that as the rate becomes doubled a

decrease in the size of the potentials of the fibers already active is produced; and the bringing into activity of new fibers at the same time the potentials in the old ones are depressed complicates the potential wave form in an uninterpretable way. To obviate this difficulty in the later

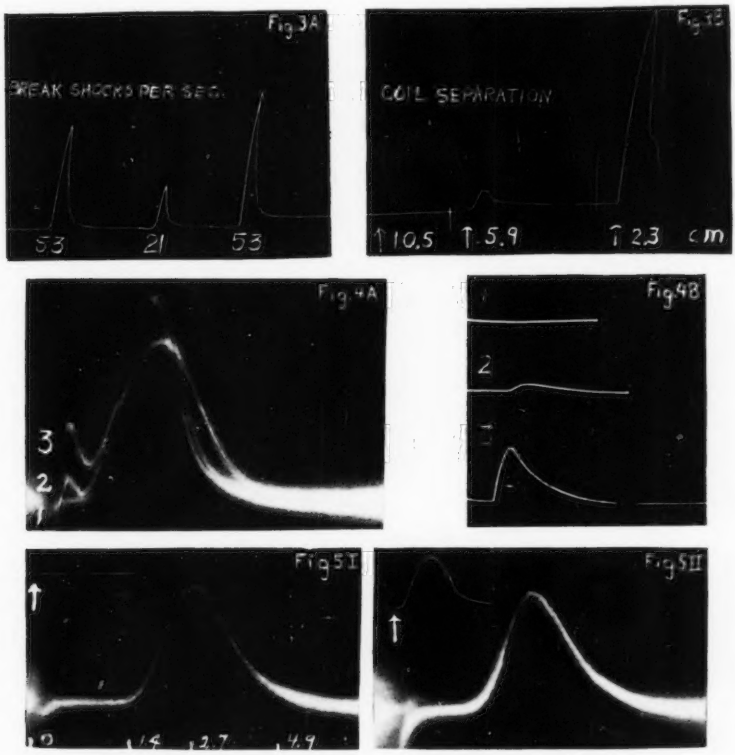

Fig. 3. Effect of rate and strength of stimulation.

Fig. 4. March 20, 1928. Stimulus 30 break shocks per second. Distance of conduction 34 mm. A: Electrical record, 1 mf., 5000ω. B: Mechanical record; *1*, subminimal; *2*, supraminimal; *3*, submaximal.

Fig. 5. January 25, 1928. Stimulus 31 break shocks per second. Distance of conduction 4.8 cm. Electrical records 1 mf., 5000ω. Time in sigmata. Insets: mechanical records; I, subminimal; II, about maximal.

experiments the make shocks were eliminated by short circuiting the secondary coil by means of a second wheel synchronized with the rotating interrupter. The fixed rate of thirty break shocks per second was arbitrarily chosen for most experiments because it is the maximum speed of our interrupter. It gave very satisfactory if not maximal responses.

RESULTS. As the strength of stimulus was increased, as is usual, the action potential increased in height and became longer. Up to the time that the area of the potential wave was nearly maximal, the spread of the stimulus, displacing the response toward the shock artefact due to the lesser distance of conduction in the faster and more irritable fibers was slight; but with strong shocks the spread was often very considerable. Moreover even with the employment of break shocks alone, before the muscle responded, a change took place in the form of the potentials produced by the fibers already active. The whole wave became slightly prolonged and decreased in height and this alteration augmented as the strength of the shock was increased (fig. 4 A; 3 as compared with 1), presumably due to the electrotonic effect on the nerve of the strong shocks themselves. In any case this factor so interfered with the orderly addition of potential areas to the wave, as described above, that it prevented the exact location of the fibers responsible for the muscle contraction.

When the stimulus was increased in size and the nerve action potential from the largest shock, which was subminimal for the muscle, was compared with one which was well above threshold, no significant difference between the action potentials could be discerned. Examples of such cases are shown in figures 4 and 5. In the experiment recorded in figure 4, when the strength of stimulation was increased from subminimal (1) to supraminimal (2) there was no change that could be measured in the form of the potential wave. There was simply an increase in the spread bringing the wave from the stronger shock earlier in the record. When the mechanical response was large, confusing secondary effects appeared (3). In the experiment of figure 5 there was a larger distance of conduction and also less spread of excitation. However, in changing from subminimal to about maximal excitation no definite change could be ascertained in the potential records although these were carefully plotted on a large scale in rectangular linear coördinates and superimposed. The results just described can only mean that the effect was due to direct stimulation of the muscle by escape of current or that the fibers involved produced too small an area in the total wave to be visible. The first possibility was eliminated by Sherrington who had his electrodes 20 cm. away from the muscle. We have measured the actual spread of stimulus along the nerve when the muscle response was positive and have found a value of 1.2 cm. for the most irritable fibers. As the electrode distance from the muscle was 2.8 cm., it is inconceivable that the stimulus could have spread to the muscle so as to stimulate it directly; and this conclusion is borne out by the fact that the response of the muscle had qualities differing decidedly from those of a response to a direct stimulus.

In as much as in nerve activity the individual fibers are added in the order of their size, as the strength of stimulation is increased the only conclusion possible is that the Sherrington phenomenon is mediated by fibers

of such very small size that they contribute so little to the area of the potential sign of the wave of the nerve's activity as to permit the latter to come to full value, as far as measurements are possible, before the fibers in question are stimulated.

Discussion. The possibility that it is the intrafusal fibers which are responsible for the contraction is definitely disproven by the fact that the tensions recorded in Part I are too large to be so explained. Furthermore, the electrical potential records prove that the nerve fibers to the spindles cannot be concerned in any other way, since they are large fibers (3) and produce an area of potential time at the front of the action wave (10). This conclusion is further justified by the fact that the lingual nerve, stimulation of which produces the tongue phenomenon, does not carry the the proprioceptive impulses.

The one positive clue to the nature of the fibers is that they are small and have their trophic centers in the dorsal root ganglia. The evidence (3), (4) has been cited to show that these fibers are not of thoracolumbar sympathetic origin. They cannot have cells of origin in the spinal cord and pass out over the posterior roots because they remain undegenerated after section of the posterior roots. There are many small fibers in the dorsal roots and a large number of them have been shown by Ranson (11) to be unmyelinated. The histology has recently been reviewed by Hinsey (12). He finds in muscles, whose ventral root and sympathetic innervation had degenerated, small myelinated and unmyelinated fibers of dorsal root origin. From the observations on the action potential, it is just such fibers that one would expect to be involved. They do not, however, end hypolemmally in muscle but are found ending in the adventitia of the blood vessels and in the connective tissue between the muscle fibers. This means that, if they are concerned—and one can hardly admit from the potential records that any other fibers are concerned—their action is indirect.

Theories of indirect action started with Heidenhain. The phenomenon seems everywhere to be associated with vasodilatation but it was proven by Heidenhain to be independent of vasodilatation per se, not however, of vasodilator fibers. The question then arises as to how these fibers might be involved. Langworthy (13) has suggested that the action currents of fibers might stimulate the hyperirritable degenerating muscle, but this hypothesis is rendered very improbable by the consideration that the large fibers to the spindles, with their much larger action currents, do not do so, and by the fact that the contraction does not have the characteristics of one produced by electrical stimulation. It has been stated that this type of contraction is caused by impulses travelling over fibers which end in the accessory terminations of Boeke (8), (13). However, these endings in muscles of the limb are said to belong to thoracolumbar sympathetic fibers and, if so, they could not be the ones involved.

The only other indirect connection between the blood vessels and the muscles which suggests itself is a chemical one. If the vasodilatation

itself should take place according to Loewi's (14) humoral transmission theory, it is conceivable that the intermediate substance might also be pharmacodynamically active on skeletal muscle sensitized by denervation, although not so on a normal muscle.* Such an hypothesis is not contrary to what is known about the sizes of the innervating fibers in the two cases. The fibers involved in the Sherrington phenomenon could not be identified beyond the determination of the fact that they are small; but even this degree of identification is sufficient to permit the association of the contracture producing fibers with those carrying impulses for dorsal root dilatation, because it has been possible to locate the latter in the 3–5μ region (15).

If Boeke's theory hold, or Langworthy's hypothesis of stimulation by the action currents of the nerves, one would expect wave-like activity in the muscle and action potential of the tetanic type. However, the action potential seems to be wave-free, just as in many of the drug contractures. Rogowicz (16) found that the Vulpian contracture in the tongue occurred without a sound corresponding to the stimulation frequency and, as would be expected from this observation, Schaeffer and Licht (17), working with the string galvanometer, obtained a record of sustained potential, which was wave free. They also reported a similar result when a sensitized leg was made to contract as the result of stimulation of dorsal root fibers

In one experiment only, 12 days after cutting the spinal roots, we made leads to the oscillograph from the gastrocnemius muscle during stimulation of the sciatic nerve. During the contracture no waves of potential appeared corresponding to the stimuli. A similar lead made from the normal side during indirect stimulation gave a very definite wave corresponding to each induction shock, even when the stimuli were so weak that the muscle produced a tension equivalent only to that of the denervated muscle, i.e., when the tension was produced by a few active muscle fibers distributed in a mass of inactive ones which would therefore act as an effective shunt. The conditions of the experiment were not such as to permit one to ascertain whether or not there was a sustained potential.

III. Antagonism by adrenalin. Adrenalin seems to have a generally depressant action on the contractures of mammalian muscle, as far as these have been studied. The contracture of the tongue produced by lingual stimulation was found by Frank, Nothmann and Hirsch-Kaufmann (5) to be prevented by adrenalin and the latter also found that the drug contractures in both the tongue and the denervated limb were similarly

* This interpretation was presented by Bremer and Rylant, "Leur effet pseudomoteur, comme leur effet vaso-dilatateur résulte probablement, ainsi que le suppose Langley (9), de la libération, au niveau de leurs terminaisons, de substances qui n'impressionnent la fibre musculaire striée que lorsqu'elle est sensibilisée par la dégénérescence." Compt. Rend. Soc. Biol., 1924, vol. 90, p. 985.

depressed (5), (18). This fact was confirmed by Gasser and Dale (19). A similar antagonism was found by Schäffer (20) for Tiegel's contracture produced in man. As no observations have been recorded of such an antagonism during the contracture produced on stimulation of the dorsal root fibers, a number of such observations were made.

METHOD. In cats, in which the gastrocnemius had been sensitized to and prepared for observation of the Sherrington phenomenon, 1 cc. of adrenalin chloride was injected into the external jugular vein. The Sherrington contractions were observed before and after the injection, using the method described in Part I.

RESULTS. In seven cats, it was found that the Sherrington contractions, which had been present before the injection of adrenalin, were inhibited afterward. This antagonism was present from ten to twenty minutes following the injection and then the contraction began to appear again. In figure 6, the maximal tetanic contraction was recorded at 12:44, and

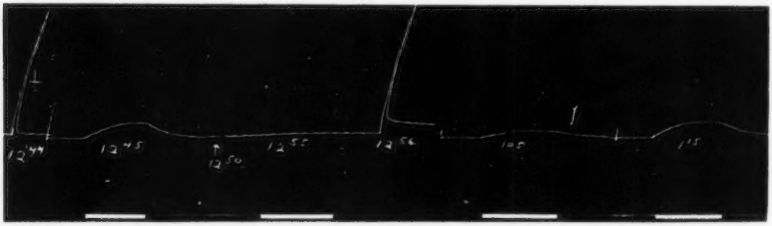

Fig. 6. Cat 34. May 2, 1928. Right dorsal and ventral roots, 6–7 L and 1–2–3 S were cut between the spinal ganglia and the cord. June 2, 1928, the experiment was performed. 12:44, maximal tetanic contraction; 12:45, Sherrington contraction; 12:50, 1 cc. of a 1/1000 solution of adrenaline chloride was injected into the external jugular vein; 12:55, Sherrington phenomenon was absent; 12:56, maximal tetanic contraction; 1:05, Sherrington contraction; 1:15, Sherrington contraction.

one minute later the Sherrington phenomenon. At 12:50, 1 cc. of a 1/1000 adrenalin chloride solution was injected into the external jugular vein. At 12:55, it was impossible to obtain an indirect contraction but the maximal tetanic contraction recorded one minute later was approximately equal in tension to the one recorded at 12:44. The Sherrington phenomenon again appeared at 1:05 and five minutes later it was equal to the pre-adrenalin value. Another cubic centimeter of adrenalin was injected at 1:25 with a repetition of the effect.

DISCUSSION. In the references cited above there is convincing evidence that the adrenalin effect is not secondary to its vaso-constrictor action but is directly on the muscle itself. It seems to have an analogy with adrenalin actions elsewhere, since it is antagonised by ergotoxin (19). When the antagonism is to a chemical stimulant there is always the possi-

bility that the mode of action is by the prevention of fixation of the drug by the cell and the far greater effectiveness of adrenalin when injected before the stimulant supports this view. However, whether or not a failure of fixation is concerned in the drug antagonisms, we must admit, in order to explain the antagonism to the Tiegel phenomenon, the existence also of a direct or indirect action on muscle metabolism preventing the particular form of energy liberation occurring in a contracture. Therefore, in as much as the antagonism exists to contractures of different origin, one can draw no conclusion from the fact of the antagonism as to the mechanism of the stimulation.

SUMMARY

The sensitization of mammalian skeletal muscle to the Sherrington phenomenon and to contracture with acetyl-choline depends upon section of somatic motor fibers. Its occurrence is not prevented by the presence of dorsal roots connected with the spinal cord or by the presence of the thoracolumbar sympathetic supply. (The finding as to the sympathetic system confirms Van Rijnberk.)

A study of the action potentials of the residual dorsal root fibers in the sciatic nerve after degeneration of the motor fibers demonstrated that the phenomenon is mediated by the small-sized afferent fibers.

In the one experiment in which it was tried, stimulation of the nerve did not lead to the production of potential waves in the muscle of a frequency corresponding to the rate of stimulation, although the muscle was in a state of contracture.

The contraction induced by stimulation of the dorsal root fibers is prevented by adrenalin.

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ULTRA-VIOLET WAVE LENGTHS VALUABLE IN THE CURE OF RICKETS IN CHICKENS[1]

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It seems necessary to know the exact region of the light and radiation spectrum which is valuable in the prevention and cure of disease. It may be possible that the region differs for different diseases. The present problem is concerned with the effect of specific wave lengths on rickets.

Ordinary window glass transmits radiations as short as 3300 Å, but absorbs shorter wave lengths; thus it prevents the healing power for rickets possessed by the sunlight. The shortest ultra-violet radiations of the sun are absorbed by the air. Sunlight cures rickets, and it is evident that radiations in the relatively narrow region, between 2900 Å and 3300 Å, are anti-rachitic. They are called the "vital rays." There is no good reason for believing that window glass cuts off these radiations just at the dividing line where the "vital rays" begin. And incidentally, if the curative rays do not include wave lengths as long as 3126 Å, it becomes a much more difficult task to make substitutes for window glass because the shorter radiations are much more readily absorbed by them. Just where the rays which possess the curative properties begin, and how far down the spectrum they extend, is still an unsettled question. In northern latitudes where the rays of winter sunshine reach the earth after passing in a slanting manner through the air, glass and glass substitutes which will transmit a high per cent of the beneficial rays, and which have the other qualities of durability and cheapness, seem to be under certain conditions, greatly needed. If winter sunshine is to be of much benefit to us, in the cure of rickets, we must have practically 100 per cent of its ultra-violet. Indeed to depend on a poor window glass substitute and expect the sunshine which passes through it to cure rickets, tuberculosis, or other diseases, might postpone other treatment at a great risk to the patient.

Bundesen (1927) and his co-workers have studied the ultra-violet spec-

[1] Aided by a fund from the Corning Glass Works. This company also prepared the filters used in the experiment.

Filters tested in the Department of Physics, Cornell University, under a grant from the Heckscher Research Fund (see Appendix).

trum of sunlight at Chicago, from November to May, and find that the shortest detectable rays are between 2990 Å and 3150 Å; usually being slightly longer than 3000 Å. The intensity of these rays is also much reduced during these months. Hess and Weinstock (1923) found that wave lengths longer than 3240 Å have no curative power in rickets. They were of the opinion that the strong band, found in the mercury vapor spectrum occurring at 3024 Å, is of great value in this respect. In a later paper these investigators conclude that there is probably a feeble curative value in the radiations from the mercury arc at 3126Å (1925). It should be noted also that Hess and Anderson (1927) now believe that "radiations shorter than those produced by the sun exert a more intense anti-rachitic effect than the most potent region of the solar spectrum."

Helborn (1927) and co-workers believe that in order to obtain a high yield of vitamin D in irradiated ergosterol, only rays longer than 270 $m\mu$[2] should be used, because of photodecomposition of the vitamin by the shorter rays.

Fosbinder, Daniels and Steinbock (1928) in a study of the photochemical activation of sterols, believe that only waves shorter than 313 $m\mu$ and longer than 257 $m\mu$ are effective in this activation. Of the lines investigated in this study, 265 $m\mu$ was most effective. If there is a difference between the activation of sterols and the living animal it is perhaps due to the covering of the latter by layers of tissue which prevent many of the radiations from reaching the blood.

The object in the present experiment was to determine as nearly as possible the specific radiations responsible for the cure of rickets. Glass filters of different kinds and thicknesses were used as a means of absorbing from the spectrum of a quartz mercury vapor arc, different wave lengths; so that some of the groups of animals were exposed to little ultra-violet shorter than 3000 Å, while the remaining groups had more and more of the rays between 2800 Å and 3000 Å; and those irradiated under the open arc had all of the rays emitted by this source.

Chickens were used as the experimental animals and the test was made by curing rickets. Chickens furnish convenient animals for such work; first, they do not depend upon milk for food in early life and therefore the food can be controlled from the day of birth, and second, rickets can be uniformly produced in practically all of the animals in spite of an abundant supply of calcium and phosphorus. On a diet containing all of the necessary food substances for normal growth in sunshine, 90 per cent of a flock of chickens, after 17 to 40 days from hatching, will develop rickets in the absence of the "vital rays." The time when symptoms appear depends upon a number of factors among which are, the food of

[2] 270 $m\mu$ = 2700 Å.

the hens before and at the time the eggs are produced, and the amount and intensity of sunshine to which the hens are exposed in the weeks just previous to the production of the eggs. If the food of the hens contains cod-liver oil or some other source of vitamin D, rickets in the chicks will be delayed. The same is true if the hens have had sunshine or artificial ultra-violet. This delay, in our experience, has been from one to three weeks. A third consideration in favor of chickens is that large numbers of animals of exactly the same age and breeding can be obtained.

We used the cure of rickets rather than prevention, because in tests on the cure of the disease the extremes can be discarded before treatments begin. In other words, those animals which develop the disease most slowly, as well as those which come down first and have the extreme early symptoms, can be eliminated. This leaves a more uniform and a much more satisfactory group with which to work.

Our animals were single comb white Leghorns, purchased from the Poultry Department of the New York State Agricultural College. Data on the breeding, feeding and care of the mother stock were available and care was taken to have vigorous, well bred chicks.

The experiment was planned to have ten pens of animals with ten chickens in a pen. Accordingly 157 chicks were bought—the 57 extra allowed for loss and culling. When the pens were finally made up, six weeks after hatching, 125 chickens were alive. Thirty-two had died or had been used in making x-ray pictures and blood tests. Twenty-five chickens were eliminated, most of them showing only mild symptoms of rickets, a half dozen had very extreme symptoms and were therefore discarded.

Symptoms. The symptoms of rickets are sometimes very marked almost from the time they are first observed. Such chickens develop a severe case of "leg weakness" without previous noticeable signs of the disease. At other times the symptoms come on more slowly. Leg weakness is usually the first striking sign and is followed by a rapid slowing up of growth, roughened feathers, and a condition of general unthriftiness. The animals have tenderness of the joints shown by the position of the feet which are extended forward along the sides of the body while the chickens are sitting. This causes the animal's weight to rest upon the breast and hocks instead of the feet. Occasionally the animals lie on their sides. In this case they have great difficulty in eating, but deformities are not so marked. The gait of rachitic chickens is halting and evidently painful and difficult. They soon fail to move about except when very hungry and this results in undernutrition. Unless care is taken on the part of the attendent there is likely to be considerable loss in the flock because of starvation. If the food and water are supplied regularly in low, easily accessible containers, and provided also that the chicks are

watched and compelled to go to the food regularly, there is little reason why they should die from rickets, at least not for six weeks after severe

Fig. 1. Recovery from extreme rickets under ultra-violet treatment. Seven minutes' irradiation per day.

A. Chick 226, mature, seven months old. The same chicken is seen on the right in B, when he was 10 weeks old and had been treated for ten days; also shown beneath the arrow in C, when he was eight weeks old and had severe rickets.

B. These chickens were the same age, both had severe rickets when three weeks old. They were raised in the same pen and had the same care, except no. 384, at the left, began receiving ultra-violet treatments four weeks before no. 226 received them.

C. The appearance of chickens with extreme rickets. Picture taken under the ultra-violet lamp after the irradiated controls had been cured of rickets.

Fig. 2 Fig. 3 Fig. 4

Fig. 2. Extreme rickets in chicken five weeks old. Dissection to show especially the beading of ribs and the bending of the breast. Observe that the ends of the femur, tibia, and tarsometatarsal bones are indistinct.

Fig. 3. Typical early rickets in chicken four weeks old. The animal showed marked external symptoms. The distance between the bones in the knee is large but the ends of the bones are still clearly seen. The epiphyses in the hock are small and far from the diaphyses. Ribs show beading.

Fig. 4. Normal chicken, same age as in figure 3. Note the denser bone, the shorter distance between bones in the joints, and the clear, well-formed epiphyses.

symptoms set in. Even then it is doubtful if chickens die from the direct effects of rickets. Their lowered metabolism, and the greatly reduced

muscular activity, along with the low blood content in at least part of the necessary elements, namely, calcium and phosphorus, predispose the animals to a number of common ailments, one of which is rhinitis. Chilling may, and often does, cause the death of rachitic birds. The body temperature is abnormally affected by changes in room temperature. Care must be exercised to keep the temperature of the animal room at a uniformly high level (75°F.) and the brooders must be still warmer (80–95°F.).

A study of the animal before and after it is killed reveals many conditions which are constant and reliable and can be used in forming a judgment regarding the severity of rickets. The photographs found in figure 1 show the appearance of very rachitic chickens and the same chickens after different periods of exposure. Interference with bone growth as a consequence of rickets results in deformities found principally in the legs and the breast. Often there is also a bending downward of the spinal column in the region of the last dorsal vertebrae, and an abnormal downward curve in the sacral and coccygeal region The neck is sometimes stiff, being fixed in the position assumed by the sitting chicken. This ankylosis is caused by a loss of elasticity and flexibility of the ligaments in the neck. The animal may have difficulty in lowering the head to the food and may be entirely unable to turn it around to the body. The legs are sometimes much bent, and abnormal deposits of limy material may cause peculiar shapes of the long bones, but usually, the bending here is not extreme. The principal abnormality is the failure of mineral deposition, the ends of the bone being mostly cartilage while the shafts become thin and fragile.

The greatest deformities occur in the ribs and breast. The latter is usually bent. There is often an acute V shape bend to right or left in the keel somewhere near its middle. An extremely bent breast means a reduced thorax and no doubt consequent pinching of the vital organs. Figure 2 shows some of these deformities, particularly in the ribs and breast.

The ribs are usually much deformed. Characteristic beading results in four rows of distinct enlargements, two found at the junction of the dorsal and ventral portions, a position comparable to the costo-chondral joints in man, and two rows where the ends of the ribs articulate with the vertebrae.

There is often a pronounced denting inward of the ribs at this joint causing a distinct groove noticeable on the outside of the body and resulting in great protrusion inward, caused by the fact that the knobs or beads are turned inward. This of course seriously reduces the size of the thoracic cavity. The constant weight of the body on the breast causes this bending of ribs and probably also the bending of the breast bone itself. The

TABLE 1

Growth records

The weight of each chicken at the beginning of the treatment and at the end of each week, also the condition of each animal at the beginning and end of the irradiation period is given.

CHICK NUMBER	BEGINNING	1ST WEEK	2ND WEEK	3RD WEEK	4TH WEEK	BEGINNING	END
Pen 1. No irradiation							
302	217	229	210	199	200	Severe rickets	Very severe rickets
205	267	282	284	285	283	Severe rickets	Very severe rickets
214	306	316	337	317	316	Severe rickets	Very severe rickets
263	290	306	329	325	325	Severe rickets	Severe rickets
217	278	287	305	292	294	Very severe rickets	Very severe rickets
213	245	255	257	258	246	Severe rickets	Very severe rickets
210	308	366	420	463	470	Moderate rickets	Severe rickets
346	298	306	312	311	301	Severe rickets	Very severe rickets
216	232	236	255	263	269	Severe rickets	Very severe rickets
378	184	193	193	185	181	Severe rickets	Very severe rickets
224	268	315	337	319	322	Very severe rickets	Very severe rickets
Ave.	263	281	294	292	291		
Pen 2. Irradiated under window glass, 21 minutes 40 seconds daily							
241	209	210	208	195	193	Severe rickets	Very severe rickets
249	323	343	356	352	338	Moderate rickets	Severe rickets
254	237	271	283	299	292	Severe rickets	Very severe rickets
251	383	412	447	475	480	Moderate rickets	Severe rickets
324	294	348	392	417	441	Mild rickets	Severe rickets
258	222	284	329	366	398	Severe rickets	Severe rickets
268	215	245	263	263	244	Severe rickets	Very severe rickets
276	318	343	335	325	300	Severe rickets	Very severe rickets
Ave.	275	307	326	336	335		
Pen 3. Irradiated under filter E for 10 minutes 20 seconds daily							
303	215	241	308	402	490	Severe rickets	Normal
322	227	225	239	273	343	Severe rickets	Moderate rickets
454	317	313	318	309	366	Very severe rickets	Rickets
325	274	285	292	327	409	Severe rickets	Mild rickets
335	253	229	271	350	450	Severe rickets	Mild rickets
260	340	382	538	688	787	Severe rickets	Normal
327	285	278	276	295	397	Rickets	Mild rickets
282	306	339	367	436	515	Moderate rickets	Normal
475	287	320	393	464	530	Rickets	Normal
476	257	306	351	398	424	Rickets	Normal
Ave.	276	292	335	394	475		

TABLE 1—*Continued*

CHICK NUMBER	BEGINNING	1ST WEEK	2ND WEEK	3RD WEEK	4TH WEEK	BEGINNING	END
Pen 4. Irradiated under filter A, 2 mm. for 2 minutes 55 seconds daily							
247	260	271	314	402	505	Severe rickets	Normal
402	369	264	302	332	430	Severe rickets	Rickets
405	227	265	277	274	317	Severe rickets	Severe rickets
408	177	179	190	205	250	Severe rickets	Rickets
401	338	354	355	375	420	Severe rickets	Mild rickets
329	283	303	360	450	547	Moderate rickets	Normal
406	335	383	491	646	778	Severe rickets	Normal
266	309	330	339	319	363	Moderate rickets	Moderate rickets
460	261	288	338	424	515	Severe rickets	Mild rickets
410	173	216	274	347	446	Severe rickets	Mild rickets
Ave.	263	285	324	377	457		
Pen 5. Irradiated under filter C, 4 mm. for 21 minutes 40 seconds daily							
467	264	265	285	283	297	Severe rickets	Very severe rickets
362	231	240	248	250	260	Severe rickets	Very severe rickets
474	283	293	287	290	282	Severe rickets	Very severe rickets
470	232	229	241	235	245	Severe rickets	Very severe rickets
301	209	209	210	207	206	Severe rickets	Very severe rickets
424	226	253	282	299	312	Severe rickets	Severe rickets
404	213	232	250	256	268	Severe rickets	Very severe rickets
458	263	263	274	257	252	Rickets	Severe rickets
418	283	273	281	283	296	Severe rickets	Very severe rickets
280	339	336	361	366	412	Severe rickets	Severe rickets
Ave.	254	259	272	297	283		
Pen 6. Irradiated ½ minute. No filter							
446	212	208	212	231	278	Very severe rickets	Severe rickets
420	187	183	191	270	333	Severe rickets	Normal
447	285	286	323	340	407	Severe rickets	Rickets
457	340	366	446	554	657	Rickets	Normal
442	274	278	294	305	373	Severe rickets	Moderate rickets
452	340	374	504	588	644	Moderate rickets	Normal
Ave.	273	282	328	383	449		
Pen 7. Irradiated 1 minute daily. No filter							
425	177	200	296	410	505	Very severe rickets	Normal
304	195	190	223	302	372	Severe rickets	Normal
422	243	249	204	393	502	Severe rickets	Normal
429	277	295	310	328	402	Severe rickets	Mild rickets
430	258	310	421	509	597	Severe rickets	Normal
421	231	249	370	496	574	Severe rickets	Normal
427	217	344	422	545	671	Moderate rickets	Normal
432	252	253	302	359	428	Severe rickets	Mild rickets
431	355	430	525	614	686	Moderate rickets	Normal
Ave.	256	280	353	439	526		

TABLE 1—*Concluded*

CHICK NUMBER	BEGINNING	1ST WEEK	2ND WEEK	3RD WEEK	4TH WEEK	BEGINNING	END
Pen 8. Irradiated 1 minute, 33 seconds, filter E, 4.5 mm.							
365	200	210	214	253	307	Very severe rickets	Rickets
343	215	219	235	254	296	Very severe rickets	Severe rickets
336	228	234	277	356	471	Severe rickets	Normal
352	199	198	200	220	240	Very severe rickets	Severe rickets
356	253	265	314	423	534	Severe rickets	Mild rickets
221	316	382	472	575	678	Rickets	Normal
240	330	363	390	455	530	Severe rickets	Normal
466	321	347	422	557	667	Rickets	Normal
Ave.	258	277	316	387	465		
Pen 9. Filter D, 5 mm., 12 minutes 20 seconds daily							
481	314	308	313	323	335	Severe rickets	Severe rickets
234	196	200	198	217	230	Very severe rickets	Very severe rickets
340	288	291	320	362	400	Severe rickets	Rickets
290	300	316	328	338	333	Severe rickets	Rickets
285	320	324	313	292	305	Severe rickets	Severe rickets
332	275	285	320	352	401	Severe rickets	Moderate rickets
261	271	275	313	385	462	Severe rickets	Moderate rickets
269	333	333	356	396	450	Moderate rickets	Mild rickets
273	279	278	335	452	544	Severe rickets	Mild rickets
453	350	383	438	512	593	Severe rickets	Normal
Ave.	293	299	323	363	405		
Pen 10. Ten minutes' irradiation without filter, daily							
222	195	245	328	444	529	Very severe rickets	Normal
357	200	215	247	338	423	Very severe rickets	Normal
444	276	320	280	503	552	Severe rickets	Normal
229	208	402	494	642	739	Severe rickets	Normal
236	257	332	413	541	638	Severe rickets	Normal
235	282	347	420	550	647	Moderate rickets	Normal
227	282	349	443	574	654	Severe rickets	Normal
286	261	314	398	534	606	Severe rickets	Normal
215	332	381	434	544	632	Rickets	Normal
237	217	241	300	422	539	Very severe rickets	Normal
Ave.	261	315	386	509	596		

deformities are due to the great weakness of bones caused by the lack of mineral matter.

Growth records are valuable in determining the rapidity and amount of recovery. While some animals grow much faster than others under the

same treatment, averages of a number of animals give fairly reliable comparisons. See tables 1 and 5.

Chickens with severe rickets frequently show considerable enlargement of the joints. This condition becomes more and more noticeable for several weeks. The bones appear to move apart, the skin and other tissues press inward between them and the joints are no longer firm, but wobble when the chicken walks. In the feet the joints are often puffy and the toes crooked and long, often bending inward. The claws and upper mandible grow slender and long and hooked from disuse, and the color of the skin as well as the color of the comb and wattles is pale and lifeless.

ROENTGENOGRAMS. X-ray pictures reveal many differences between the normal and the rachitic bone. In early rickets there is still a clear shadow across the ends of the bones much like the normal. There seems to be a band two or more millimeters wide where inorganic matter is still being deposited. This becomes narrower and narrower until it finally disappears leaving the ends of the bone with an irregular frayed-out appearance. Figures 2, 3 and 4 show x-ray photographs of rachitic and normal chickens. Bone differences are easily distinguished.

At the beginning of the irradiation period x-ray pictures were taken of the right hock joint of all of the chickens. Another similar photograph was taken about two weeks later. These proved very satisfactory and useful in comparing the process of bone changes in the different pens, and in watching the development of osseous tissue in the individual chickens. At the end of the experiment roentgenograms were made of the two halves of each chicken. The animals were carefully skinned, all of the viscera removed and the carcass split down the right side of the spinal column.

After the disease has been marked for two or more weeks the ends of the long bones often become indistinct. The distal end of the femur appears blurred, the proximal end of the tibia is often wide and cup-like with an irregular notched rim. What seems to be actual excavations of bony material appear in the distal end of the tibia. See figure 2.

IRRADIATIONS. Treatment of the different groups of chickens was as follows:

Pen 1 received no irradiation. Pen 2 was treated under a filter made from ordinary window glass, filter D. Pen 3, irradiated under filter F, a green glass having a thickness of 7.2 mm. Pen 4 received daily exposure under filter A, a clear glass, 2 mm. thick. These clear glasses transmit almost all of the visible rays. Pen 5, filter C, 4 mm. thick, is a clear glass. Pen 6 had 30 seconds' exposure each day without a filter. Pen 7 had 60 seconds' without a filter. Pen 8 was irradiated under filter E, which is a dark purple glass transmitting much ultra-violet but a limited amount of the visible rays. Pen 9, filter B, 5 mm. thick, was from the same material as the filter used in pen 4. Pen 10 was irradiated without a filter and received ten minutes' exposure daily.

The controls of the experiment were: pen 1 without irradiation, pen 6 with 30 seconds', pen 7 with 60 seconds', and pen 10 with 600 seconds' daily exposure.

Table 2 shows the total transmission of filters A, B, C and D for the time the respective groups were irradiated beneath them. It shows also the relative units received by the chickens irradiated under the open arc. Filters E and F became less transparent to short rays after continued exposure to ultra-violet. This disappointing characteristic is possessed by many of the new substitutes for window glass.

Irradiation began on June 18, 1927. The room in which the work was conducted was 20 feet by 30 feet with an 8 foot ceiling. It was dry and well ventilated but lighted entirely by Mazda lamps. Heat for the brooders in each pen was furnished by two 40 watt Mazda lamps. The

TABLE 2

Total transmission of filters

In per cent, based on unit exposure of 1 minute. (Open arc)

	WAVE LENGTH								RECOVERY
	2,755 Å	2,804 Å	2,894 Å	2,926 Å	2,968 Å	3,024 Å	3,126 Å	3,324 Å	
Open 60 seconds.....	100	100	100	100	100	100	100	100	95
Filter A.....			20	33	59	100	170	246	78
Open 30 seconds.....	50	50	50	50	50	50	50	50	67
Filter B.....				9	32	105	370	940	48
Filter C.....					12	50	292	1,170	8
Filter D.....								346	0
Open 10 minutes.....	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	100

light from these lamps shone directly upon the chickens beneath the brooder.

Experience had convinced us that one minute exposure to such a source and intensity of ultra-violet as used here, was about the minimum necessary to bring about satisfactory cure of rickets. It seemed also certain that ten minutes' exposure was far more than necessary for very rapid, possibly maximum rate of cure. Likewise we believed that 30 seconds was less than was necessary and would give only partial cure in four weeks time. This is the time required under favorable conditions, to effect a cure by the quartz mercury arc treatment.

The source of ultra-violet radiations was a direct current Uviarc 110 volt Cooper Hewitt quartz mercury arc. The current was developed in the irradiating room adjoining the animal room by a motor generator producing a current of 110 volts. The potential across the lamp was adjusted to 80 volts by a permanent setting of the series ballast. Slight variations from this voltage were controlled by adjustments in a ventilating fan.

The animals were irradiated in light wooden boxes of rough boards, twenty inches wide, twenty-five inches long and eighteen inches deep. When placed beneath the mercury arc with the filters in place, all of the light fell within the box but struck high up on its sides and ends. The distance from the floor of this box to the burner was $22\frac{1}{2}$ inches. A large sheet of heavy colored paper was slipped beneath the opening in the housing when the different groups were being changed. In this way the exposure could be accurately timed. The order of treatment was rotated so that a group that was irradiated first one day, came second the next, etc. No irradiation was begun until the voltage reached 80 across the arc. This required about seven minutes.

FOOD AND CARE OF ANIMALS. For the first three days food of the chickens consisted of rolled oats. After that it was made up of a dry mash plus night and morning feedings of a standard scratch mixture. The formulae of the food mixtures were as follows:

Mash	*pounds*	*Scratch*	*pounds*
Dried buttermilk	200	Cracked corn (yellow)	1200
Meat scrap, 55–60%	200	Cracked wheat	800
Wheat flour middlings	300		
Fine ground oats	100		
Corn meal (yellow)	680		
Wheat bran	400		
Bone meal, steamed	100		
Sodium chloride	20		
Total	2000		

After the first week the mash was kept constantly before the chickens. Previous to this the feed pans were placed before them five times per day for intervals of increasing length. This prevented over-feeding, a dangerous thing for young chickens. The same procedure is often necessary in the case of supplying water. One principal object in making up the ration was to supply an abundance of phosphorus and calcium. Five per cent of the mash was ground bone, and 10 per cent dried buttermilk. The aim was to supply a diet that is complete in all of the necessary constituents except sunshine or vitamin D and one that had proved to be, in earlier experiments, entirely adequate for growth under normal conditions.

We believe that to cut down the variety, or to simplify too much the food of young chickens, leads to complications including undernutrition, and brings about a picture not wholly characteristic of rickets.

At first our chickens grew rapidly. While they were a little under average size at hatching time, during the first four weeks they grew considerably faster than their fellows under ordinary poultry farm conditions. After this the chickens that were out of doors rapidly overtook them in weight.

Table 3 gives the average weights, week by week, and the average gains of the 150 chickens up until the end of the sixth week when treatments began. Gains were normal until the end of the third week. The approach of rickets evidently caused a slight slowing up of growth in the fourth week, and at the end of the fifth week gains had been much reduced. The average for the seven days of this week was 17 grams, a typical "rachitic gain." Most of the animals were showing marked signs of the disease by then. It is somewhat remarkable that in the next week the gains were much better. We had noticed earlier that birds seem to rally from the first severe onset of the disease as if there were a mobilization of the last reserves.

TABLE 3

Growth of chickens during development of rickets

	AT HATCH	1ST WEEK	2ND WEEK	3RD WEEK	4TH WEEK	5TH WEEK	6TH WEEK
Average weight in grams....	33	69	110	163	211	228	266
Gain.....		36	41	53	48	17	38

TABLE 4

Weight of cockerels selected from Cornell Poultry Farm flocks June 12, 1927

	AGE							
	3RD WEEK	4TH WEEK	5TH WEEK	6TH WEEK	7TH WEEK	8TH WEEK	9TH WEEK	10TH WEEK
Weight (average)....	90½	124	274	368	465	695	716	987

Table 4 gives the average weights of two cockerals for each age selected from flocks of the same breeding as the experimental chicks but kept under normal out-of-door conditions on the poultry farm.

Table 5 gives the weight of the separate pens at the beginning of irradiation and the average gain per week per chick for the four weeks of irradiation. Pens 1, 2 and 5 made rachitic gains for a time but at the end of the experiment were all losing weight. The chickens in pen 10 began improving almost immediately after the irradiations began. There was an average gain of 54 grams in the first week. After that time gains averaged about 90 grams per animal per week which is normal for maximum recovery of animals six weeks old. Pen 7 showed no noticeable acceleration in growth the first week, but some improvement in general appearance was seen. After this, gains in weight for pen 7 were about as good as for pen 10. Between the gains of pens 10 and 7 and the rachitic gains of 1, 2 and 5, the amount of growth of the remaining pens depended upon the quantity of ultra-violet obtained by each group, of a very limited wave

length. The stimulation of the organism to recovery, as a result of irradiation seems also to be cumulative. When the dose is small a week or more elapses before growth is accelerated.

In order that we might have a point of attack we assumed line 3024 Å in the mercury vapor spectrum to be a very important one in the cure of rickets. If this line, with its high intensity of radiant energy, should prove to be the region of the most beneficial rays, then by giving a number of the groups the same exposure to it, results should be about the same. On the other hand, if there developed marked variations in these groups the differences would likely be due to variations in amount of exposure from other regions. Pens 4, 7 and 9 had the arbitrary unit (100 per cent)

TABLE 5

Growth of chickens under treatment (six weeks old at beginning)

PEN NUMBER	AVERAGE WEIGHT AT BEGINNING	GAIN DURING				FILTERS AND AMOUNT OF EXPOSURE
		1st week	2nd week	3rd week	4th week	
	grams	*grams*	*grams*	*grams*	*grams*	
1	263	18	14	14	−1	No irradiation
2	275	32	19	10	−1	D. 21 minutes 40 seconds
3	276	16	43	59	77	F. 10 minutes 20 seconds
4	263	22	39	53	80	A. 2 minutes 55 seconds
5	254	5	13	25	−14	C. 21 minutes 40 seconds
6	273	9	46	55	66	Open arc 30 seconds
7	256	24	73	86	87	Open arc 60 seconds
8	258	19	39	71	78	E. 1 minute 35 seconds
9	293	6	24	40	42	B. 12 minutes
10	261	54	71	123	87	600 minutes open arc

exposure to this wave length. This was the amount received by the one minute open arc (pen 7) group. One half of this unit was received by the chicks in pen 6, which had one-half minute irradiation. It was necessary to increase the time of exposure of pen 4, filter A, to two minutes fifty-five seconds, and the time of pen 9, filter B, to twelve minutes twenty seconds in order to obtain this unit of exposure to line 3024 Å. Pen 5 received 50 per cent of the unit exposure to this line. Actual exposures to shorter waves were below the unit for all of the chickens irradiated beneath filters except in the case of those treated under filter E, but of the longer waves all of the other groups had much more than the amount received by the one minute group. The author was aided in this part of the work by Mr. J. L. Wierda who made the chemical tests on blood.

The entire blood of six average animals from each group was collected. These separate samples were allowed to stand twenty-four hours and then measured amount of serum taken from each and mixed with a like amount

from the other sample belonging to the same group. From this composite sample of serum the test was made. Fisk and Sabbarow's method for determination of inorganic phosphorus, and the Clark-Collip modification of the Kramer-Trisdall method for serum calcium were used. Table 6 gives the results of chemical analyses of blood and bone.

The right femur bone was used for ash determinations. When all of the muscle and the soft tissues, except the periosteum, were removed the weight was taken and recorded as "green bone." The dry weight was obtained by drying at a temperature of 110°C. until no further loss occurred. The organic substances were burned out in a Muffle furnace at a temperature of 700°C. for two and one-half hours or until there was no further loss. In this way the weight of "total ash" was obtained.

TABLE 6

Results of blood and bone analyses

PEN	FILTER	Ca	P	Ca × P*	BONE ASH	RECOVERY
					per cent	*per cent*
1	No irradiation	7.65	2.76	21.11	9.15	None
2	Filter D	7.52	2.78	21.00	9.61	None
5	Filter C	7.00	4.34	30.38	10.91	8
9	Filter B	9.11	4.34	39.13	13.07	45
6	30 seconds open arc	8.51	4.30	37.55	14.46	67
8	Filter E	8.71	4.65	40.89	15.05	74
4	Filter A	9.24	4.82	45.57	16.06	78
3	Filter F	9.17	4.88	44.10	15.00	82
7	60 seconds open arc	10.30	4.82	49.24	17.53	95
10	600 seconds open arc	10.56	4.60	48.30	16.29	100
11	Sunlight	11.35	5.71	64.41	15.41	100

* The product obtained by multiplying the units (milligrams per 100 cc. blood serum) of calcium by the units of phosphorus.

Our final score on the different pens was made up from a careful study of 1, general appearance; 2, growth weight curves; 3, post-mortem examinations; 4, roentgenograms; 5, blood analysis for serum calcium and phosphorus, and 6, total ash of the bone.

In comparing the recovery of the different groups with the irradiation received by each group, from the respective lines of the mercury arc spectrum, the order of recovery corresponds to the total quantity of radiation (time and intensity) of wave length 2968Å. It does not in any way correspond to the amount of radiation from wave length 3024Å. There are eleven distinct lines and bands in the quartz mercury arc spectum beginning at the group of lines at 2537Å, and ending at 3342Å. The relative amount of the radiations from each of these bands, transmitted by the different filters, in the respective time the corresponding groups were

treated, is given in table 2. It will be noted that between wave length 2968Å and 2804Å there are but two lines, 2926Å and 2894Å; the former is faint, the latter is bright and has an intensity of approximately one-third that of wave length 2968Å. This, no doubt, has considerable power in the cure of rickets. Only four lines (3024, 2968, 2926 and 2894Å) seem to have any noticeable effect in the cure of rickets in chickens. Those longer seem ineffectual, those shorter seem to be obstructed by the superficial layers of the skin.

If we assume that wave lengths 3024Å and 2968Å are important, then filters C group could not have received any appreciable benefit from the longer wave lengths, because these chickens received 50 per cent of the standard exposure to the line 3024Å, and more than 10 per cent exposure to line 2968Å, and secured only slight benefit (8 per cent). This does not take into account the longer wave length ultra-violet coming from the Mazda lamps. Other groups bear out the same conclusion. For example, filter D group received almost 400 per cent the standard exposure of wave lengths 3126Å, more than 100 per cent standard exposure of wave length 3024Å, and 30 per cent of a standard exposure to line 2968Å. They were benefited less than 50 per cent.

Line 3024Å has about double the intensity possessed by the next line, 2968Å. We are led to conclude that line 3024Å cannot possess more than a moderate curative power. This is shown in comparing the radiations and benefits received from lines 3024Å and 2896Å by the groups under filters A and B. See table 2.

The filters were tested before the irradiations began and again immediately at the close of the work. It was found that two of the Corex glasses, E and F, had changed during the test and had become less transparent to the ultra-violet. This, of course, makes any exact statements relative to the quantity of irradiation from them impossible. However, the change took place particularly in the region below 2900Å and it might be assumed that the change came about gradually, therefore, as the tables were made from the transmission at the close of the test, the animals in these groups must have had more exposure to those shorter waves, rather than less than is shown.

We believe that the experiment shows that rays shorter than 2894Å have little curative value. The chickens in pen 3, filter F, had considerable exposure to the rays beginning at 2654Å, and were not enough improved over those of pen 4, filter A, which transmitted no wave lengths shorter than 2894Å, to indicate that these shorter rays had any effect at all. Similarly, if the shorter rays were markedly valuable, pen 6 (30 seconds open arc) receiving 50 per cent of the unit exposure to them, should have been nearer cured than pen 4, filter A, which had none. Out of the radiations shorter than those at 2968Å only one seems to be of much

importance. This is the 2894Å line. Those radiations at 2926Å are too feeble to cause much impression upon the photographic plate by which method the intensity of the rays is measured. This region must also have a comparatively low power in the cure of rickets.

We believe also that radiation at 2968Å is perhaps the center of the most important region in the mercury arc spectrum involved in the cure of rickets. The radiation of wave length just longer (3024Å) has perhaps one-fourth the total value of line 2968Å.

SUMMARY

A comparison of different wave lengths as to their power to cure rickets, was made by using glass filters capable of absorbing different quantities of the shorter ultra-violet.

After producing rickets in 125 chickens the flock was culled, eliminating the least rachitic and those most affected. Ten groups containing approximately ten chickens in a group were thus obtained. With these the test was conducted.

Those wave lengths effective in the cure of rickets are in the region between 3130Å and 2650Å. Those shorter than 2896Å seem, from our work, to be relatively weak in this respect; and line 3130Å seems to have no curative value.

Careful analysis of the data of this experiment seems to prove conclusively that wave lengths 2968Å are the most potent in the cure of rickets and that line 3024Å is perhaps one-fourth as effective.

APPENDIX

Determination of Transmission of Glasses

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In view of the purpose of the present investigation to obtain quantitative data, both on the effective wave lengths in the ultra-violet which cure rickets and on the quantity of radiation necessary with the animals under consideration, viz., chickens, it was essential that accurate measurements of the transmissive power of the glass filters used should be made. In the matter of the filters, as two pieces of each were used in the irradiating apparatus care was taken to observe that they were of uniform thickness, with well polished surfaces, and that both had the same transmission.

For the determination of the ultra-violet transmission of the various

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filters, the apparatus consisted of a Hilger quartz sector photometer in conjunction with a Hilger quartz spectrograph, E36. A discussion of the principles underlying this method of measuring transmission has been published by Howe (Phys. Review, 1916, viii, 674) and by Gibson and others, Bureau of Standards Scientific Papers, no. 440 (1922). Briefly, it consists in taking two beams of light from a single source; one traverses the specimen and a revolving sector wheel of fixed angular opening, the other beam is diminished in intensity by decreasing the angular opening of a second revolving sector wheel. The light source used was a discharge under water of a Tesla coil, electrodes of brass or tungsten being employed. By placing the electrodes 12 to 16 mm. apart and using the center of the gap, a perfectly continuous spectrum is obtained without any of the characteristic line radiations of the materials of the electrodes. The continuous spectrum extends to the limit of transmission of our spectrograph—2150 Å. The two beams are brought into juxtaposition by means of a bi-prism set before the slit of the instrument and it is possible to make twelve to fifteen exposures on a single plate, each corresponding to a different ratio of the sector openings. The per cent transmission is computed from the ratio of the angular openings of the two sectors. From the photographic plate the wave lengths are located at which equal blackening occurs on the pair of contiguous spectra corresponding to each value of the per cent transmission. In the case of the colored filters, a Schmidt and Haensch spectrophotometer equipped with a Lummer-Brodhun contrast prism was used for measuring the transmission in the visible part of the spectrum.

The results were first plotted in the form of transmission curves, with per cent transmission as ordinates and wave lengths as abscissae, but for ease in interpreting the final results curves showing the relative total transmission as a function of the irradiation times were constructed. The times of irradiation through the various filters were so chosen as to give approximately the same amount of energy of the Hg line, 3024Å, through each of the filters. These times were calculated by assigning a unit time of exposure to the above wave length and dividing this time by the per cent transmission at that wave length for each filter. While this desired result was not actually achieved, particularly in the case of filters C and E, the subsequent findings of the investigation showed that there was no need for such a uniformity at wave length 3024Å, since the most active radiation physiologically turned out to be 2968Å.

Having settled on an irradiation time for each filter, the transmission curves were obtained in the following manner: The time for a given filter was multiplied by the per cent transmission corresponding to each of a number of wave lengths. This operation thus gave the effective amount of irradiation in seconds for these wave lengths. This number was then divided by 60, the time in seconds which had been found sufficient to

produce almost complete cure of rickets, when the chickens were exposed to the bare mercury arc for this length of time daily. In this way it was possible to read off directly the per cent irradiation, based on the standard of one minute exposure of the open arc, for any filter at any particular wave length. Thus, for example, filter A was found to transmit 25 per cent at 2990Å and its irradiation time was 175 seconds. The effective amount of energy at this wave length corresponded therefore to 44 seconds of the open arc or to 73.3 per cent of that obtained by the one minute, open arc group.

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SOME CHEMICAL CHANGES IN MUSCLE PRODUCED BY DRUGS[1]

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THE EFFECT OF DRUGS UPON THE INORGANIC CONSTITUENTS OF THE SMALL INTESTINE. A great deal of work has been performed upon the action of ions on excised tissue and it is now recognized that salts or their ions play an important part in the function of tissues. The action of the ions may be separated into two parts, first their osmotic action and second, their specific ion action. The osmotic action has not been particularly studied in this investigation, although its effects have probably been influencing the results to some extent, but rather the specific ion actions of sodium, potassium, calcium and magnesium have been considered.

Sydney Ringer (23) did most of the fundamental work on the specific ion actions in preparing a perfusion fluid and since his time numerous investigators have continued to study this subject. Howell (9) suggested from the similarity of the action of potassium and that of the vagus nerve stimulation on the heart that the action of this nerve might depend on the setting free of potassium in some manner. Howell and Duke (10) found an increase of potassium in a small amount of Ringer-Locke's solution which was repeatedly perfused through a mammalian heart under vagus inhibition. Hemmenter (7), however, was not able to detect any difference in the potassium content of the ash of normal and inhibited hearts.

Neuschlosz (19) working on heart muscle has shown from analyses of fresh tissue and water extracted tissue that some potassium exists in a bound and some in a free condition. Using the drug, acetyl choline, he found the fractions affected in the following way:

	NORMAL HEART	0.85 PER CENT KCl	ACETYL CHOLINE
	mgm. per cent	*mgm. per cent*	*mgm. per cent*
Total potassium	640	890	320
Bound potassium	240	150	160

[1] An abstract of a thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Pharmacology in the Graduate School of the University of Illinois, 1929.

The work of Howell and Neuschlosz suggested that the action of drugs might take place through changes in the various inorganic constituents and so the present investigation was undertaken.

Methods. The small intestine of the dog was used because one can easily obtain adequate samples for analysis—a normal segment may be removed, and then the drug injected and a second sample taken. The action of the drugs used on the intestine of the dog is also well known—atropin and adrenalin causing a relaxation and pilocarpine a constriction. The analyses were made upon a trichloracetic acid extract because it was thought that any changes which might take place would be more likely to be noted. This extract would contain all of the elements which were ionized and also the fractions which were in a rather loosely bound organic form.

Dogs were used in all the experiments. They were taken from the stock pen, anesthetized with ether and their abdomens shaved, washed, and then opened. A piece of the small intestine weighing about 25 grams was removed by tying off the blood vessels to the segment and ligaturing both ends. The mesentery was removed as much as possible. The drug used was then injected into the femoral vein and after several minutes when its action could be detected, another sample of the intestine was removed in the same manner as the first. This sample, however, usually consisted of part of the gut above and another below the normal specimen in order to avoid as much as possible any differences in composition of the intestine at various levels. Both specimens were placed in beakers as soon as removed and prepared for analysis at once, generally within five minutes from removal of the first specimen. Blood was removed from the outside of the gut by means of filter paper. The gut was then opened longitudinally, the submucosa stripped off, and the whole specimen cleaned with filter paper. The sample was then ground up and a part of it weighed into a crucible for the moisture determination. The remainder, about twenty grams, was used for the analysis.

Moisture was determined by drying at 105° for several days or until a constant weight was obtained and the percentage of water calculated.

The tissue to be analysed was placed in a wide mouth glass stoppered bottle and 35 cc. of 20 per cent trichloracetic acid added. After thorough mixing the material was allowed to stand for about two hours with frequent shaking. At the end of this time the solution was filtered into a 50-cc. volumetric flask, the extraction bottle and the material remaining on the filter paper were washed several times. The filtrate in the flask was made up to the mark and the aliquot parts of the solution used for the various determinations. The calcium and magnesium were determined according to the method given by Kramer and Howland (14), the potassium and sodium according to the method given by Kramer (13).

The results have been calculated in milligrams of element per 100 grams of dried tissue. It was thought that the water content of the tissue might be changed by the drugs and so apparently influence the results. Further calculations were made to determine the changes in the ion balance of the tissues.

The doses of drugs injected were not always the same. Usually a small dose was given and if it produced no observed change in the intestine, a second dose was injected. The amounts given came within the following range:

	mgm. per kilo of body weight
Pilocarpine	3–4
Atropin	0.1–0.2
Adrenalin	0.1–0.2

Results. Ten experiments were completed using atropin, twelve using pilocarpine and eight using adrenalin. The individual experiments are quite consistent and so only the summaries are given in the table.

No effort has been made to obtain absolute values for the various constituents so that results as given are merely relative. In fact, Whelan (26) reports that there is a positive error of from 3 to 5 per cent in the determination of sodium, potassium and calcium, while with magnesium it may be as high as 15 per cent. Since the analyses of the normal and after drug specimens in each experiment were run simultaneously it was thought that any errors inherent in the method would be cancelled.

No calculations are given of the percentage change of the elements, i.e., of the percentage difference between the normal and after drug figures. The results which are important appear more clearly when considered from the actual change—either a decrease of (−) or an increase (+) of the after drug values in comparison with the normal values.

The results show that pilocarpine produces a decrease of all the elements while atropin produces the opposite—an increase in the various constituents. The results produced by adrenalin are varied—the potassium and magnesium are decreased while the sodium and calcium are increased.

Discussion. Considering first only the results obtained with the parasympathetic drugs we see that stimulation of this nervous system with pilocarpine produces a decrease of the acid extractable fraction of the elements, while inhibition of this nervous system with atropin causes an increase of these same elements. Whether these two types of reaction are due to the stimulation or inhibition of the nervous system by the drugs or whether they are due to the change in the state of the muscle—contracted or relaxed—produced by the drugs cannot be determined from the investigation.

Any attempt to explain the changes or the cause of the changes produced

by pilocarpine and atropin is of doubtful value because there are so many factors which must be considered and about which information is lacking. However, the results seem to agree with some of the facts that have been published. It is known (5) that hypotonic solutions produce increased tone and excitability on excised tissue. Further it is natural to suppose

TABLE 1

	MEAN VALUES (MGM. OF ELEMENT PER 100 GRAMS OF DRIED TISSUE)			IONIC CONCENTRATION: MEAN VALUE / ATOMIC WEIGHT OF ELEMENT	
	Normal	After drug	Difference	Normal	After drug
Experiments with atropin					
H_2O	79.1	79.5	+0.4		
Mg	71.5	77.0	+5.5	2.94	3.17
Ca	30.1	31.2	+1.1	0.75	0.78
K	1362.0	1487.0	+125.0	35.0	37.3
Na	262.5	337.5	+75.0	11.4	14.7
Na/K				0.33	0.39
K/Ca				46.5	47.8
Experiments with pilocarpine					
H_2O	79.97	79.07	−0.90		
Mg	82.1	72.0	−10.1	3.34	2.96
Ca	33.95	33.15	−0.8	0.85	0.83
K	2019.0	1845.0	−174.0	51.6	47.2
Na	182.0	149.0	−33.0	7.91	6.47
Na/K				0.15	0.14
K/Ca				60.9	57.0
Experiments with adrenalin					
H_2O	80.66	80.5	−0.16		
Mg	86.25	81.87	−4.38	3.54	3.36
Ca	36.75	42.38	+5.63	0.92	1.06
K	1640.0	1588.0	−52.0	41.9	40.6
Na	252.3	268.0	+16.0	10.9	11.6
Na/K				0.26	0.29
K/Ca				45.6	38.2

Note: The water content is given in terms of per cent.

that tissues placed in hypotonic solutions will lose some of their inorganic elements. Pilocarpine is known to increase the tone and excitability. In the present work, a decrease of the various elements in the tissue was produced by pilocarpine.

Hypertonic solutions cause a depression of excised tissue and with this statement a similar method of reasoning can be used for the results ob-

tained with atropin. Just how valid the argument is, is doubtful because there are many other factors connected with osmotic pressure relations which have not been studied or controlled in the present work, but which were nevertheless affected by the drugs.

Considering the calcium and magnesium ions and their effect on smooth muscle, it is stated (24) that an increase of either produces a depression of both muscle and nerve and the increased amount of the elements found in the atropin experiments in the present study agrees with this observation.

It has been shown that the action of physostigmine is antagonized by calcium (18) and this also probably holds for pilocarpine. Joseph and Meltzer (11), (12) have shown the same to be true for magnesium. In the experiments with pilocarpine in this investigation, the calcium and magnesium are both decreased, which would favor the action of the drug.

Concerning the potassium/calcium ratio, it is still more doubtful if any interpretation can be given to the values found in this work. Generally it is thought that in heart perfusion experiments with an increase of the potassium/calcium ratio the effects resemble a parasympathetic stimulation. In the present experiments with atropin and pilocarpine the ratios are exactly the reverse. Just how to explain this is rather difficult. Hogben (8) reports that with a low potassium/calcium ratio and an increase in acidity, he obtains an increased muscular tone. According to Evans (6) there is a formation of lactic acid in contraction of plain muscle. Taking the work of these investigators into consideration one might expect a lowered potassium/calcium ratio after pilocarpine as was found in this study. However just what one should say about the atropin potassium/calcium ratio is problematical.

Howell and Duke (10) report that they found an increase in the potassium content of the perfusion fluid of a heart stimulated by the vagus nerve. This would indicate that there was a loss of potassium from the heart muscle. Neuschlosz (19) finds a decrease in the free and bound potassium of the heart perfused with Ringer's solution containing acetylcholin. The present work with pilocarpine is in agreement with these investigators. In considering the inhibition of the heart, however, there are probably other factors to be considered than the specific effect of potassium. Loewi (17) finds that when the heart of a frog is inhibited by stimulation of the vagus, a substance is given off into the perfusion fluid which when circulating through another heart has the power of inhibiting it. Loewi points out, however, that the activity of this inhibitory substance is suspended by atropin, which is not the case in pure potassium inhibition. Loewi's work has been confirmed by Brinkman and van d. Velde (3), Witanoski (27) and Popper and Russo (22).

The results obtained with adrenalin are rather difficult to understand

in relation to the results with atropin. Both drugs produce a relaxation of the intestine. Atropin acts by inhibiting the parasympathetic nervous system allowing the sympathetic system to have greater influence. Adrenalin acts by an active stimulation of the sympathetic system. Whether this difference in method and site of action of the drugs is sufficient to explain the varied results has not been proved as yet but it is given as a suggested means of accounting the contrast in results. Atropin caused an increase in all the constituents, while adrenalin caused an increase in calcium and sodium and a decrease in magnesium and potassium.

The osmotic pressure relations which were noted in the pilocarpine and atropin experiments do not hold true for the adrenalin experiments.

That there is a causal relation between calcium and adrenalin has been advanced by several investigations. The vaso-constrictor action of adrenalin on the arterioles of the frog was shown by Pearce (21) to be converted into a dilator one in the absence of calcium. Bayliss (1) could not confirm these results. He found that the constriction was still produced by adrenalin after long perfusion with calcium-free Ringer's solution although the addition of calcium increased the effect of adrenalin to a small degree.

Libbrecht (16) found that the adrenalin acceleration on the heart is hindered by excess or absence of calcium although potassium has relatively little influence.

Burridge (4) found that the absence of calcium tends to render adrenalin depressant.

Lange (15) found that adrenalin has the power of decreasing the permeability of muscular interfaces and in this way preventing edema and inhibition of water by the fibre.

Osterhaut (20) found that all substances that affect permeability belong to one of the two classes which antagonize each other:

1. Sodium chloride, potassium chloride type causing an increase in permeability.

2. Calcium chloride type causing a decrease in permeability up to a certain concentration, beyond which they act like sodium chloride.

Beattie and Milroy (2) have observed that in minced muscle, adrenalin decreases the rate of "floride" synthesis of lactic acid to hexophosphate.

The decrease of the potassium/calcium ratio resulting from adrenalin agrees with the usual conception this carries, i.e., a stimulation of the sympathetic nervous system.

CONCLUSIONS

This investigation shows that the trichloracetic acid extractable inorganic elements of the small intestine are affected by the drugs pilocarpine, atropin and adrenalin in the following way:

1. Pilocarpine produces a decrease in the sodium, potassium, calcium and magnesium.

2. Atropin produces opposite changes from pilocarpine—an increase in the sodium, potassium, calcium and magnesium.

3. Adrenalin produces an increase in the sodium and calcium and a decrease in the potassium and magnesium.

Whether these changes are myogenic or neurogenic in origin cannot be determined from this investigation.

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THE DEVELOPMENT OF SECONDARY SEX CHARACTERS IN CAPONS BY INJECTIONS OF EXTRACTS OF BULL TESTES[1]

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The finding of a fraction of the lipoids extracted from bull testicles which produces growth of the comb, wattles and ear lobes in brown leghorn capons when injected in suitable amounts was previously announced (McGee) in the Proceedings of the Institute of Medicine of Chicago in April of last year (1). The details of the original investigations on extracts of bull testicles are outlined in a doctor's dissertation submitted to the Department of Physiological Chemistry and Pharmacology of the University of Chicago in September, 1927 (2). The majority of the attempts at assay of such extracts were made by observing secondary sex characters in castrated cockerels (capons) during a series of injections of the material. Studies on the influence of the extracts on the size of the seminal vesicles of castrated guinea pigs after injections are summarized in the dissertation thus: "Individual variations in such a heterogeneous group of guinea pigs render less significant such conclusions which one might draw from the comparison of seminal vesicles of the castrated animals under various conditions. About ninety guinea pigs were used in the attempts to demonstrate an effect of the lipoid fraction "X" on these structures of the castrate. . . . . The average size of the seminal vesicles of a treated castrate is larger than the average of that of an untreated castrate or that of the animals receiving the lipoid fraction obtained from calf thymus. The difference, though definite, is not large." For a more significant attempt at assay of the material on mammals examine the paper by Moore and McGee appearing in THIS JOURNAL. The material presented in the present paper has to do with observations made on the extraction, chemical treatment and assay on capons of our extract.

The dependence of the secondary sex characters upon the presence in the organism of functional gonad tissue and by inference, upon the products

[1] The expenses of this investigation were supported in part by the Committee for Research in Problems of Sex of the National Research Council. The grant is administered by F. R. Lillie.

elaborated by this tissue, are discussed in a large number of special publications as well as in representative textbooks. In the survey of the literature given here we will limit ourselves to examples of the action of gonad extracts on birds and to some examples of the methods of extraction applied to testis tissue.

It is difficult indeed to evaluate properly the literature appearing on methods of extracting activity from gonads. Little worthy effort at a chemical study seems to have been made in the case of the testes. The findings here are not as clear as those obtained after extirpation or transplantation of testicular tissue. Obviously, the secret of the difficulty in attempting to study such gland extracts (as it has been in the history of the biochemical investigation of other tissues, glands or biological material) is the want of methods of assay. The lack of justifiable criteria has in the past invalidated much scientific study.

Brown-Sequard (3) in 1889 initiated the interest in the internal secretory value of the gonads by injections into himself of testicular tissue extracts. The experimenter was seventy-two years of age. The observations were purely subjective and have not been uniformly confirmed by other investigators.

Poehl (4) isolated spermine as a phosphate. It is an organic base claimed by him to be the main endocrine product of the organ and to have the power to accelerate oxidation in the living tissue or to act as a carrier of oxygen. These claims have not been substantiated. The product has been shown to exist in localities other than in the testicle, viz., in the nervous tissues and indeed, according to Dixon (5), in all body tissues. The most recent contribution to the knowledge of spermine is the demonstration of its structural formula and the verification of this by chemical synthesis. The formula ascribed to it is $NH_2(CH_2)_3NH(CH_2)_4$-$NH(CH_2)_3NH_2$. It is the first instance of a base of this type, containing the trimethylenediamine group, found in nature (6).

Bouin and Ancel (7) reported that the effects of castration on the skeleton and the genital organs of adult guinea pigs can be stopped by subcutaneous injections of a glycerol-water extract of the testicles. They stated that extracts of testicles of larger mammals as well as the extracts of testicles of the same species are effective on guinea pig castrates in inducing masculinity. Injections of such extracts into young guinea pigs appeared to accelerate the growth.

Walker (8) in 1907 found that a normal saline extract of fresh, macerated cock's testicles injected in one-half cubic centimeter quantities, subcutaneously, each day for several months in two hens caused a marked growth of the comb and wattles. After the procedure was stopped the combs and wattles regressed so that the original size finally was approximated.

Iscovesco (9), after a detailed study of lipoids in tissues, extracted from both ovaries and testicles fractions exhibiting interesting activities. He announced the finding of a lipoid fraction from the testicle showing activity which appears to be similar to that expected of the chemical furnished by the testicle in the form of an internal secretion. This was part of the lipoid material obtained from dried testicular tissue with hot ether. Injections into normal rabbits (a) stimulated "rutting characters," (b) "excited the kidney," (c) "excited the spleen," and (d) "excited the testicles" (the kidney and spleen and testicle increased in weight as shown by Iscovesco's figures during the injection procedure), and (e) accelerated growth in young and augmented weight in adult rabbits. Some clinical applications of the preparation in cases of obscure ailments of men gave results interpreted as being positive for "internal secretory" benefits of the lipoid fraction. The authors have noted no published repetition of the findings of Iscovesco by other workers.

In 1911 Pezard (10) injected into a young black Orpington capon twice each week, over a period of more than three months, 5 to 10 cubic centimeters of an extract of the testicle from a cryptorchid hog. He reports that under the influence of the injections the comb and wattles became thick, hyperemic, erect and larger in size. Furthermore, sexual instincts appeared such as crowing and combative attempts with other males. After the injections were stopped the bird resumed the characters and behavior of a typical capon.

Ssentjurin (11) in 1926 attempted to demonstrate activity in perfusates from bull and stallion testicles. The perfusion mixture was a "Ringer-Locke's" solution warmed to 38°C. The fluid in large amounts was injected four times daily under the skin of mature cockerels soon after castration. He showed inhibition of the rate of regression of the comb of the birds receiving the perfusate. Ssentjurin claimed that the rapid decolorization of an alkaline solution of "Lichtgrün" was characteristic of the active isotonic solutions. This phenomenon was paralleled by a decrease in the amount of work performed by the muscle of an isolated frog-heart suspended in such a perfusate as he used.

Aude (12) attempted to employ the lower basal metabolism of castrated cockerels as compared to normal cockerels as an assay criterion for extracts of testes of both bulls and cockerels. He extracts aseptically macerated testes with serum and filters this through glass wool. Injections are made into the comb or into the peritoneum of the capon. An increased carbon dioxide output is found. Slight inhibition of the rate of regression of the comb following post-puberal castration is reported to have been obtained from these injections.

Busquet (13), using senescent cockerels, injected blood serum from young bulls, stallions and rams, and concluded that a return of male secondary

sex characters is observed in the assay cockerels. Administration per os of the serum above mentioned to the old cockerels likewise was effective in Busquet's opinion.

Korenchevsky (14) gives detailed methods for the isolation of lipoid fractions of the prostate and testes which, when administered together, were in his opinion effective in raising the nitrogen metabolism in rabbits. He reports that "none of the fractions isolated from the prostate or testes injected singly or mixed in the doses given, is able to prevent the atrophy of the penis, seminal vesicles, and prostate of castrated rats or is able to produce hypertrophy of the genital organs of normal rats." In a second paper (15) the same investigator reports negative effects upon nitrogen metabolism from the "water soluble, protein-like" substances obtained from aqueous extracts at varying hydrogen ion concentrations by precipitation with sodium chloride or by converting certain extractable substances into hydrochlorides.

METHODS. Allen and Doisy and collaborators (16), Frank and Gustavson and co-workers (17), and others during the past decade have shown the applicability of common fat solvents to the extraction of follicular fluid, corpus luteum and placenta in deriving a lipoid mixture containing one or more activities usually designated in current scientific literature as the "female sex hormone." Acting upon the possibilities suggested by these findings we have determined that lipoids showing the property of stimulating the growth of the comb, wattles and ear lobes in the capon can be derived from bull testicles. The method of extraction used by R. G. Gustavson for ovarian studies has been applied in our work most uniformly in preparing stock benzene solutions for further experimentation and attempts at purification. The fundamental procedure described by E. A. Doisy for obtaining follicular fluid extracts has also been successfully applied to testicular tissue as shown below.

Because of the accessibility of the slaughter houses in Chicago relatively large quantities of testes from bulls of all ages were obtained during the course of the work. For several months about seventy pounds of frozen testicles, stripped of the epididymides, were received at the laboratory each week. The material was prepared for extraction in the following manner: Testicular tissue was stripped of tunics, ground and placed in twice its weight (two and one-half volumes) of ninety-five per cent ethyl alcohol at room temperature. The material was mixed frequently during the succeeding forty-eight to seventy-two hours; afterward the alcohol was removed by decanting and by filtering through cloth. The tissue was placed under pressure in a wine press to remove remaining portions of fluid. The tissue was then discarded. The alcoholic extract was concentrated and used for the studies indicated in the several procedures outlined in this paper. The concentrated alcoholic sludge was fractionated

by brief shaking and standing with an equal volume of benzene. The benzene soluble portion is taken for further treatment.

The benzene soluble portion of the alcoholic extract from 250 kilograms of tissue received from the stock yards between June 20 and August 27, 1927 was used as a stock solution for many of the preparations. Fractions obtained from this stock solution have the letter "S" after the number designating the sample. Fractions not so marked were not prepared from this particular benzene stock solution.

The extracts of testicles were prepared for assay on brown leghorn capons by placing the lipoids in olive oil either as a true solution or as a suspension. In a few instances it was necessary to employ dilute glycerol or physiological saline solution as a menstruum. Care was used to remove as much of the solvent previously used in extracting the lipoids as was possible by vacuum distillation from a water bath. Traces of such solvents as benzene are factors in producing local irritation at the site of injection into the capon.

All of the birds used in these experiments were pure bred brown leghorns, obtained from one source and received in the laboratory as one day chicks. Caponization was done by Dr. L. V. Domm. The capons used as test animals were kept in large pens with free access to sunlight and sufficient opportunity for exercise.

Fractions to be assayed were prepared, numbered and sent to Dr. Mary Juhn who made most of the injections and observations. Subcutaneous injections were made daily except Sunday. Care was taken to use alternating sides of the capons on successive days and to distribute the injections over as large a region of the body as feasible. The non-resorbed part of the injected fractions persisted either as a yellow-brown material of the consistency of thick cream discharging through an ulcer—referred to as an "open sore," or became inspissated and surrounded by connective tissue. The latter condition is listed in the tables as "subcutaneous nodules."

Measurements of the capons' combs were taken just previous to the first injection and twice a week subsequently. The dimensions of the comb were obtained by measuring the total length of the blade from the anterior end to the posterior tip and the height by measuring one selected point, this as a rule was the barble most directly over the eye. The absolute growth in length is greater than the absolute growth in height; when, however, these increases are related to the original measurements it is found that the proportional increases, or the rate of growth in length or height are roughly comparable. The product of the length by the height (LH) is taken as an expression of comb size. An index of growth was established for ten, twenty and thirty days to facilitate comparisons of several birds by dividing (LH) at the indicated intervals by the original product of length by height (original LH). The quotient thus obtained is

employed as the measure or index of growth and as such is given in the tables.

The regression of the head furnishings after the cessation of injections occurred in every assay. Six to eight weeks or longer are required for the comb of an experimental animal to revert to approximately the original size after stopping injections of an active preparation. Of the capons not used repeatedly for assays the majority died due to one cause or another. On nine of these, autopsies were performed. None showed regenerating testes nodules in the abdominal cavity.

SUMMARIZED PROCEDURES FOLLOWED IN DETERMINING THE PROPERTIES OF THE ACTIVE AGENT. *Procedure I* (Fractions 1, 2, 9, 11, 10, 12, 39–209, 38–211, 47–333, 47–340, 51–339, 70–361, 71–362, 52–340). An alcoholic extract of ground bull testicles was concentrated to a volume one-fifteenth to one-eighteenth of the original volume by distillation under reduced pressure from a water bath at 85–90 degress Centigrade. To the warm aqueous sludge was added an approximately equal volume of benzene. After a few hours the benzene was removed from the aqueous phase and concentrated to a small volume under reduced pressure. This was prepared for injections after adding absolute alcohol and continuing distillation to aid removal of benzene.

Procedure II (Fraction 3). Method as in procedure I using freshly ground calves' thymus in place of testicular tissue.

Procedure III (Fraction 4). Method as in procedure I with the exception that the alcoholic filtrate during the concentration is heated to dryness with partial charring and extracted with benzene after cooling.

Procedure IV (Fractions 8, 13, 18, 19, 22, 23, 20, 21, 24–312, 25–313, 26–321, 26–314, 81–372). An alcoholic extract of ground bull testicles was concentrated to a volume one-fifteenth to one-eighteenth of the original volume by distillation under reduced pressure from a water bath at 85 to 90 degrees Centigrade. To the warm sludge was added an equal quantity of benzene. After a few hours the benzene was removed from the aqueous phase and concentrated to a small volume under reduced pressure. Four or five volumes of absolute alcohol were added to the extract. After standing several hours at -10 to -5 degrees Centigrade the precipitate was removed by filtration. The material insoluble in cold alcohol was prepared for assay (fraction 7). The filtrate used for assays is represented by fractions listed at the heading of the procedure.

Procedure V. The material precipitated with cold absolute alcohol in procedure IV was treated with three volumes of cold acetone. The undissolved portion was placed in olive oil for assay. Fraction 28–402.

Lipoid material separating on partial concentration of the acetone filtrate and cooling was used for assay. Fraction 27–401.

Acetone solution on further concentration and then cooling yielded crystals. These were prepared for assay. Fraction 29–319.

Procedure VI. To the concentrated benzene solution prepared in procedure I were added four to five volumes of acetone. After keeping the solution at zero degrees Centrigrade for several hours this was filtered and the filtrate taken and prepared for assay. Fractions 27–316, 37–323, 37–329, 50–338.

The precipitated material on the filter paper was treated at the low temperature indicated with a great excess of absolute alcohol. After several hours the material was filtered. The filtrate was used for assay. Fractions 28–317, 40–325.

The precipitate remaining from the above was redissolved in benzene and absolute alcohol and prepared for assay. Fractions 49–337, 76–367S.

Procedure VII. The concentrated benzene solution obtained in procedure I was extracted with liquid ammonia. Very poor exposure was obtained in this treatment.

At room temperature (under pressure): Ammonia soluble 30–501.

At room temperature (under pressure): Ammonia insoluble 31–502.

At atmospheric pressure (temperature of −38.5 degrees): Ammonia soluble 32–503.

At atmospheric pressure (temperature of −38.5 degrees): Ammonia insoluble 33–504.

Procedure VIII. The concentrated benzene solution obtained in procedure I was treated with five volumes of methyl alcohol at −10 to −5 degrees Centigrade. After several hours the material was filtered. The filtrate was prepared for assay. Fraction 34–601, 36–603.

The precipitated material was prepared for assay. Fraction 35–602.

Procedure IX. Method as in procedure I with this difference: Before the benzene layer was separated from the aqueous sludge hydrochloric acid was added (one part 36 per cent acid to one part benzene). Twenty-four hours later the benzene was removed, concentrated and prepared for assay. Fraction 41–326.

Procedure X. Method as in procedure IV except that the benzene stock solution after standing five months was used in this preparation. The filtrate after treatment with cold alcohol was used for assay. Fractions 42–327S, 42–336S, 42–352S, 83–374S, 89–380S.

The precipitated material from the above was prepared for assay. Fractions 90–381S, 91–382S.

Procedure XI. Method as in procedure I and continued: Material exposed to light at 65 to 68 degrees Centigrade for ten days. Fraction 14.

Material exposed to light with air current bubbling through the solution at 65 to 68 degrees Centigrade for ten days. Fraction 15.

Material in darkened room with air current bubbling through the solution at 65 to 68 degrees Centigrade for ten days. Fraction 16.

Material in darkened room at 65 to 68 degrees Centigrade for ten days. Fraction 17.

Procedure XII. To the concentrated benzene solution obtained as in procedure I were added four or five volumes of acetone. The material remained at room temperature for twenty-four hours. The precipitate was removed by filtration and prepared for assay. Fraction 79–370S.

The filtrate was prepared for assay. Fraction 69–360S.

Procedure XIII. The concentrated alcoholic extract obtained from procedure IV was treated with three volumes of isopropyl alcohol. The filtrate was prepared for assay. Fraction 74–365S.

The precipitated material was prepared for assay. Fraction 75–366S.

Procedure XIV. Method as in procedure IV except that absolute alcohol was added at room temperature and the material was kept at room temperature for precipitation. The filtrate was prepared for assay. Fraction 92–383.

Procedure XV. The acetone filtrate obtained as in procedure VI was absorbed on strips of filter paper and extracted with liquid ammonia in thermos bottles. Three fresh portions of ammonia were used in this extraction. The dissolved material was then prepared for injection. Fraction 62–353S.

The material remaining on filter paper strips after exposure to liquid ammonia was redissolved in benzene and prepared for injection. Fraction 53–343S.

Procedure XVI. The concentrated alcoholic filtrate obtained as in procedure X was absorbed on strips of filter paper and extracted with liquid ammonia in thermos bottles. Three fresh portions of ammonia were used in this extraction. The dissolved material was then prepared for injection. Fraction 64–355S, 84–375S.

Procedure XVII. The material found insoluble in alcohol, ether, or a mixture of alcohol and ether remaining after the concentration of a liquid ammonia extract of active lipoid material was dissolved in physiological saline. A true solution formed. This was assayed. Fractions 63–354S, 100S and 65–356S.

Procedure XVIII. An alcoholic extract of fresh tissue was concentrated nearly to dryness (with care). The residue was dissolved in dilute alkali and the aqueous solution extracted with ether. The ether solution was removed, the ether distilled off and the solids fractionated between 70 per cent ethyl alcohol and petroleum ether. Six extractions of the alcoholic solution were made with one-fourth its volume of petroleum ether.

Alcoholic solution was assayed as Fraction 54–344.

Petroleum ether solution was assayed as Fraction 55–345.

Procedure XIX. The concentrated benzene solution obtained as in procedure I was treated with six to eight volumes of seventy per cent alcohol at room temperature. This was kept at −10 to −5 degrees Centigrade for twelve hours and filtered (slow process). When the

filtrate, warmed to room temperature, was allowed to stand, two liquid phases appeared. The upper layer was a turbid fluid, light brown in color. This was concentrated for assay. Fraction 45–332.

The lower layer from the above procedure was viscid and dark brown in color. Fraction 44–331.

Procedure XX. The alcoholic filtrate obtained as in procedure IV was made a seventy per cent solution (by adding sufficient water to make thirty per cent of the final volume). This was extracted several times with fresh portions of petroleum ether. The petroleum ether extract was prepared for assay. Fraction 72–363S.

The seventy per cent alcoholic extract was prepared for assay. Fraction 73–364S.

Procedure XXI. The acetone filtrate obtained as in procedure VI was evaporated to dryness under diminished pressure and redissolved in petroleum ether. This was extracted with several portions of eighty per cent ethyl alcohol. The alcohol solution was concentrated and kept at zero degrees Centigrade for eighteen hours. The precipitate formed was assayed. Fraction 86–377S.

The filtrate from the procedure was assayed. Fraction 87–378S.

Procedure XXII. The concentrated extract from procedure I was emulsified in an excess of water and the material shaken vigorously. Lipoid in masses separated from the aqueous phase. This was removed by filtration. Lipoid on filter paper was prepared for assay. Fraction 66–357.

The filtrate from the above procedure was prepared for assay. Fraction 67–358.

Procedure XXIII. The concentrated benzene solution from procedure I was treated with N/2 sodium ethylate for eighteen hours at boiling temperature. Later the material was evaporated to a partially dry, viscid mass and treated with ethyl ether. Ether washings were prepared for assay. Fraction 56–346.

The above saponification process was repeated using N/1 sodium ethylate on an active lipoid for nine hours. The ether washings were prepared for assay in glycerol. Fraction 60–350S.

BIOLOGICAL OBSERVATIONS. The tables below summarize the biological observations made after the subcutaneous injection of the various fractions described above.

TABLES ON BIOLOGICAL OBSERVATIONS

TABLE 1

Procedure I

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
1	2 cc. daily	5- 4-27 6-16-27	1,396 1,446	4.0 7.1	1.8 3.7	7.23 26.27	1.93	2.13	2.44	3.56	586	5-23-26 (a) 8-26-26
2	2 cc. daily	5- 4-27 6- 2-27	1,338 1,404	4.0 5.9	1.3 2.5	5.20 14.75	2.02	2.28	2.84		568	5-27-27 (b) 7-27-26
9	2 cc. daily	5-26-27 6-22-27	2,042 2,028	4.7 8.0	2.5 4.5	11.75 33.88	2.26	2.85	2.88		514	6-16-26 (c) 8- 8-26
12	2-3 cc. daily	5-26-27 6-16-27	1,893 1,948	4.1 6.2	1.5 2.7	6.15 16.74	1.79	2.72			513	6-16-26 (d) 8- 8-26
11	2 cc. daily	5-26-27 6-15-27	1,863 1,875	4.2 5.8	2.0 3.0	8.40 17.40	1.70	2.07			504	6-16-26 (e) 8- 6-26
39–209	2 cc. daily	10-18-27 12-12-27		5.8 9.5	3.0 5.8	17.40 55.10	1.68	2.21	2.26	3.16	514	6-16-26 (f) 8- 8-26
38&47	2 cc. daily	10-20-27 12- 3-27	2,125 1,913	5.2 8.3	2.5 5.0	13.25 41.50	1.64	1.87	2.20	3.13	510	6-16-26 (g) 8- 6-26
51–339	2 cc. daily	11-29-27 12-15-27	1,373 1,112	5.1 7.8	2.4 4.4	12.24 34.32	1.91	2.80			903	6- 6-27 (h) 8-22-27
51–339	2 cc. daily	11-29-27 12-15-27	1,430 1,270	5.0 7.0	2.5 4.1	12.50 28.70	2.01	2.30			929	6- 6-27 (h) 8-25-27
52–340	2 cc. daily	12- 6-27 12-22-27	1,585 1,417	5.1 8.0	2.5 5.1	12.75 40.80	2.55	3.20			926	6- 6-27 (i) 8-26-27
52–340	2 cc. daily	12- 6-27 12-22-27	1,585 1,389	5.1 7.0	2.6 4.0	13.26 28.00	1.89	2.11			909	6- 6-27 (i) 8-27-27
70–361	2 cc. daily	2-28-28 3- 9-28		5.8 6.6	2.2 3.0	12.76 19.80	1.55				513	6-16-26 (j) 8- 8-26

(a) Blue-green spots, subcutaneous nodules and open sores formed.
(b) Bluish discolorations and thickenings of the skin.
(c) Blue spots developing into open sores.
(d) Skin roughened, no sores or subcutaneous nodules.
(e) Some discoloration at sites of injection, skin in fair shape.
(f) 1-22-28, capon found dead. No testis fragments. Subcutaneous material.

TABLE 1—*Concluded*

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
70–361	2 cc. daily	2-28-28 3- 9-28		5.7 6.6	2.7 3.5	15.39 23.10	1.50				624	3-22-27 (k) 6- 8-27
71–362	2 cc. daily	2-28-28 3- 9-28		5.2 6.6	2.0 2.9	10.40 19.14	1.84				42	(l)
71–362	2 cc. daily	2-28-28 3- 9-28		4.6 5.1	2.3 2.7	10.58 13.77	1.30				548	4-15-27 (l) 6- 9-27

(g) Open sores.
(h) Open sores extruding material.
(i) Capon in very poor shape, open sores extruding material.
(j) Skin in fair shape, subcutaneous lumps.
(k) Subcutaneous nodules but skin in fair shape.
(l) Subcutaneous lumps formed, skin in fair shape.

TABLE 2

Procedure II

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
3	2 cc.	5- 4-27 5-18-27	1,629 1,461	4.4 4.5	1.7 1.8						541	(a)

(a) 9-7-27. Capon dead. No testis fragments. Subcutaneous nodules.

TABLE 3

Procedure III

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
4	2 cc. daily	5- 4-27 5-18-27	1,388 1,410	3.5 3.8	1.9 2.0						565	5-27-26 (a) 7-27-26

(a) No skin reaction but capon very feeble.

TABLE 4

Procedure IV

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
7	2 cc.	5-24-27	1,530	5.2	2.1						578	6-25-26 (a)
	daily	6- 2-27	1,408	4.8	1.8							8-26-26
8	2 cc.	5-24-27	1,500	4.0	1.4	5.60					572	5-27-26 (b)
	daily	6-28-27	1,644	7.6	3.4	25.84	2.68	3.91	4.61			8- 3-26
13	2 cc.	6-20-27	1,989	6.1	2.2	13.42					513	6-16-26 (c)
	daily	7-21-27	1,960	7.2	3.2	23.04	1.45	1.74				8- 8-26
18	1 cc.	7-12-27	422	2.6	1.1	2.86					J541	4-15-27 (d)
	daily	8-10-27	533	4.0	2.3	9.20	1.85	3.22				6- 9-27
20	3 cc.	7-12-27	1,410	4.3	2.6	11.18					565	5-27-26 (e)
	daily	8-10-27	1,375	4.8	3.2	15.36	1.15	1.37				7-27-26
21	1 cc.	7-12-27	406	3.0	1.1	3.30					605	4-15-27 (d)
	daily	8-10-27	500	6.3	3.8	23.94	3.20	7.25				5- 2-27
22	3 cc.	7-12-27	1,325	4.4	2.1	9.24					568	5-27-26 (f)
	daily	8- 9-27	1,270	7.2	2.9	20.88	1.56	2.04	2.26			7-27-26
23	3 cc.	7-12-27	1,523	5.3	2.2	11.66					578	6-23-26 (g)
	daily	8- 9-27	1,561	7.8	3.8	29.64	1.73	2.48	2.54			8-26-26
24&	2 cc.	8- 1-27	1,910	6.5	2.6	16.90					24	(h)
26	daily	9-13-27	1,529	7.9	3.7	29.23	1.77	1.89				
25-	1 cc.	8- 1-27	360	3.1	1.5	4.65					500	4-15-27 (i)
313	daily	8-26-27	348	4.6	2.5	11.50	1.80	2.26	2.47			6-10-27
81-	2 cc.	4- 6-28	1,770	5.9	2.6	15.34					H541	(i)
372	daily	4-27-28	1,670	8.4	4.1	34.44	2.07	2.24				
81-	2 cc.	4- 6-28	1,495	4.8	2.4	11.52					918	6- 6-27 (i)
372	daily	4-27-28	1,485	7.5	4.3	32.25	1.97	2.80				8-24-27
19	1 cc.	7-12-27	347	5.3	2.7						633	4-15-27 (j)
	daily	8-10-27	536	7.9	4.7							Cockerel

(a) Capon in poor condition, no pronounced skin reaction.
(b) Sites of injection inflamed, subcutaneous nodules formed.
(c) Open sores and subcutaneous nodules.
(d) Skin thick and yellow.
(e) Open sores extruding material.
(f) 9-28-27. Dead. No testis tissues. Much encysted material. Sores.
(g) Subcutaneous nodules formed, skin in fair shape.
(h) Skin reaction not pronounced, capon in fair shape.
(i) No skin reaction.
(j) Veins distended, skin thick and yellow.

TABLE 5

Procedure V

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
28–	2 cc.	8–19–27	1,074	5.5	2.1	11.55					188	3–22–27 (a)
402	daily	8–30–27	1,120	5.4	2.3	12.42	1.07					6– 8–27
27–	2 cc.	8–15–27	1,106	5.0	2.6	13.00					622	3–22–27 (b)
401	daily	8–27–27	1,041	5.5	2.5	13.75	1.06					6– 8–27
29–	1 cc.	8–15–27	988	4.7	1.9	8.93					624	3–22–27 (c)
319	daily	8–30–27	1,268	4.9	2.0	9.80	1.24					6– 8–27

(a) Little skin reaction.
(b) Discolorations and open sores. Dead 8–27–27. No autopsy.
(c) No skin reaction.

TABLE 6

Procedure VI

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
27–	2 cc.	8–15–27	1,082	4.0	1.7	6.80					193	3–22–27 (a)
316	daily	9–12–27	1,176	7.4	4.0	29.60	2.73	4.35				6– 8–27
37–	2 cc.	10–20–27	1,300	4.6	2.2	10.12					198	4–15–27 (b)
323	daily	11–15–27	1,110	6.1	3.4	20.74	1.80	2.22				6– 9–27
37–	2 cc.	10–20–27	1,532	4.7	2.4	11.28					624	3–22–27 (c)
323	daily	11–15–27	1,525	8.1	4.8	38.88	2.45	3.26	3.45			6– 8–27
50–	2 cc.	11–29–27	1,025	8.4	4.2						♂50	(d)
338	daily	12– 5–27	987	8.5	4.1							
50–	2 cc.	11–29–27	1,015	8.5	5.1						♂51	(d)
338	daily	12– 5–27	992	8.6	5.3							
28–	2 cc.	8–15–27	745	4.0	1.7	6.80					621	3–22–27 (e)
317	daily	9–12–27	812	4.7	2.5	11.75	1.24	1.62	1.73			6– 8–27
40–	2 cc.	10–25–27	2,220	5.4	2.4	12.96					513	6–16–26 (f)
325	daily	12–25–27	2,115	6.8	3.2	21.76	1.14	1.46	1.68			8– 8–26
40–	2 cc.	10–25–27	1,814	5.4	2.2	11.88					42	(g)
♂325	daily	12–25–27	1,726	7.3	3.2	23.36	1.40	1.94	1.97			

TABLE 6—*Concluded*

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
49–337	2 cc. daily	1-17-28 1-27-28		5.1 4.8	2.0 2.0						904	6- 6-27 (h) 8-22-27
76–367S	2 cc. daily	4- 4-28 4-10-28		4.7 4.8	2.4 2.4						905	6- 6-27 (i) 8-22-27
76–367S	2 cc. daily	4- 4-28 4-10-28		5.3 5.5	2.6 2.9						909	6- 6-27 8-22-27

(a) 12-12-27. Dead. No testis tissue. Non-resorbed material, emaciated.
(b) Large yellow-blue spots, skin dry and hard.
(c) Large open sores formed.
(d) At sites of injection skin sloughed off.
(e) 11-9-27. Dead. No testis tissue. Much encysted material.
(f) Subcutaneous lumps and open sores. Skin recovered rapidly.
(g) Skin inflamed in spots.
(h) Skin discolored at sites of injection.
(i) No skin reaction.

TABLE 7

Procedure VII

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
30–501	1 cc. daily	8-30-27 9-13-27	848 685	4.5 4.5	2.1 2.2						195	3-22-27 (a) 6- 8-27
31–502	2 cc. daily	8-30-27 9-13-27	920 850	3.6 3.6	1.9 1.5						135	3-22-27 (b) 6- 8-27
32–503	1 cc. daily	8-30-27 10- 7-27	1,058 974	3.7 5.3	1.6 2.7	5.92 14.31	2.20		2.42		618	3-22-27 (c) 6- 8-27
33–504	2 cc. daily	8-30-27 9-13-27	892 887	4.1 4.1	2.0 2.0						617	3-22-27 (d) 6- 8-27

(a) 9-23-27. Dead. No testis tissue. Non-resorbed material, subcutaneous.
(b) 10-29-27. Killed. No testis tissue. Oily subcutaneous material.
(c) 11-22-27. Killed. No testis tissue. Oily subcutaneous material.
(d) 11-9-27. Dead. No testis tissue. Encysted material under skin.

TABLE 8

Procedure VIII

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
34–	1.5 cc.	9–23–27	1,195	5.4	2.9						188	3–22–27 (a)
601	daily	10– 5–27	1,236	5.6	2.9							6– 8–27
36–	1.5 cc.	9–23–27	1,118	3.5	1.7	5.95					548	4–15–27 (a)
603	daily	10– 7–27	1,220	4.1	2.6	10.66	1.61	1.79				6– 9–27
35–	2 cc.	9–27–27	1,424	4.7	2.4						624	3–22–27 (a)
602	daily	10– 7–27	1,482	4.2	2.4							6– 8–27

(a) No skin reaction.

TABLE 9

Procedure IX

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
41–	2 cc.	10–25–27	1,687	4.5	1.7	7.65					H541	(a)
326	daily	11–15–27	1,485	5.2	2.4	12.48	1.59	1.63				

(a) No skin reaction but capon in poor condition.

TABLE 10

Procedure X

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
42–	1–2 cc.	10–25–27	1,685	4.2	2.3	9.66					565	5–27–26 (a)
327S	daily	12– 9–27	1,594	7.7	6.3	48.51	1.91	3.02	3.89	5.02		7–27–26
42–	1–2 cc.	10–25–27	1,713	5.3	2.1	11.13					572	5–27–26 (b)
327S	daily	12– 9–27	1,670	9.2	4.7	43.24	1.44	2.69	3.68	3.88		8– 3–26
42–	2 cc.	12–13–27		4.5	2.2	9.90					586	6–23–26 (c)
336S	daily	1– 3–28		7.5	4.3	32.25	2.30	3.26				8–26–26
42–	2 cc.	1–31–28		6.5	2.8	18.20					572	5–27–26 (c)
352S	daily	2–21–28		8.5	4.0	34.00	1.27	1.87				8– 3–26

TABLE 10—*Concluded*

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
42—	2 cc.	3-14-28		5.1	2.7	13.77					586	6-23-26 (c)
352S	daily	3-27-28		6.9	3.2	22.08	1.60					8-26-26
83—	2 cc.	4-13-28	1,825	4.8	3.1	14.88					565	5-27-26 (d)
374S	daily	4-27-28	1,819	5.9	4.1	24.19	1.69					7-27-26
89—	2 cc.	5- 8-28	2,004	4.5	2.0	9.00					40—	4-15-27 (e)
380S	daily	5-25-28	2,015	5.3	2.5	13.25	1.42	1.47			27	6-10-27
89—	2 cc.	5- 8-28	1,830	4.0	1.9	7.60					42—	4-15-27 (e)
380S	daily	5-25-28	1,800	5.0	2.5	12.50	1.39	1.64			27	6-10-27
90—	3 cc.	5- 4-28		5.1	2.1	10.71					42	(f)
381S	daily	5-15-28		6.5	2.9	18.85	1.76					
90—	3 cc.	5- 4-28		4.6	2.0	9.20					931	6- 6-27 (f)
381S	daily	5-15-28		5.7	2.6	14.82	1.61					8-27-27
91—	2 cc.	5- 4-28		5.4	2.2	11.88					624	3-22-27 (g)
382S	daily	5-15-28		5.5	2.7	14-84	1.25					6- 8-27
91—	2 cc.	5- 4-28		4.5	2.3	10.35					548	4-15-27 (g)
382S	daily	5-15-28		5.0	2.5	12.50	1.20					6 -9-27

(a) Small subcutaneous lumps and open sore. Capon in good condition.
(b) Subcutaneous lumps. Capon in fair condition.
(c) Skin thickened and yellowish. Capon in good condition.
(d) Large subcutaneous lumps.
(e) Skin showed small shrivelled spots.
(f) Open sores extruding non-resorbed material.
(g) Small open sores.

TABLE 11

Procedure XI

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
14	2 cc.	6-25-27	1,495	3.7	2.1	7.77					565	5-27-26 (a)
	daily	7-13-27	1,410	4.3	2.6	11.88	1.51					7-27-26
15	2 cc.	6-25-27	1,862	5.2	2.1	10.92					504	6-16-26 (b)
	daily	7-16-27	1,670	6.7	3.0	20.10	1.87					8- 6-26

TABLE 11—*Concluded*

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
16	2 cc.	6–25–27	1,702	4.1	2.1	8.61					510	6–16–26 (c)
	daily	7–14–27	1,565	6.5	3.4	22.10	2.20	2.57				8– 6–26
17	2 cc.	6–25–27	1,714	5.1	1.9	9.69					42	(d)
	daily	7–14–27	1,587	7.8	3.3	25.74	2.23	2.66				

(a) No skin reaction.
(b) Subcutaneous lumps formed.
(c) Skin slightly inflamed.
(d) Large and deep open sore.

TABLE 12

Procedure XII

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
79–	3 cc.	4– 4–28		5.3	2.3						904	6– 6–27 (a)
370S	daily	4–10–28		5.2	2.4							8–22–27
79–	3 cc.	4– 4–28		6.5	2.3						578	6–23–26 (a)
370S	daily	4–10–28		6.4	2.3							8–26–26
69–	2 cc.	2–28–28		4.6	2.0	9.20					911	6– 6–27 (b)
360S	daily	3– 9–28		6.1	3.1	18.91	2.05					8–22–27
69–	2 cc.	2–28–28		5.2	2.7	14.04					926	6– 6–27 (b)
360S	daily	3– 9–28		7.6	4.3	32.68	2.32					8–26–27

(a) No skin reaction. Capon in rather poor condition.
(b) Deep open sores extruding waxy non-resorbed material.

TABLE 13

Procedure XIII

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth				Number	Hatched, Castrated
							10d.	20d.	30d.	Fin.		
74–	1 cc.	3–16–28		4.3	1.9	8.17					931	6– 6–27 (a)
365S	daily	3–27–28		5.5	2.6	14.30	1.75					8–27–27
74–	1 cc.	3–16–28		4.3	2.3	9.89					909	6– 6–27 (a)
365S	daily	3–27–28		5.5	3.0	16.50	1.67					8–22–27
75–	2 cc.	3–16–28		4.9	2.5						903	6– 6–27 (b)
366S	daily			4.8	2.3							8–22–27
75–	2 cc.	3–16–28		5.0	2.2						927	6– 6–27 (b)
366S	daily			4.9	2.2							8–26–27

(a) Large subcutaneous lumps.
(b) Blue-green spots and open sores.

TABLE 14

Procedure XIV

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth				Number	Hatched, Castrated
							10d.	20d.	30d.	Fin.		
92–	2 cc.	5–18–28	1,816	4.5	2.1	9.45					41–	4–15–27 (a)
383	daily	6–19–28	1,778	6.5	3.4	22.10	2.00	2.37	2.34		27	6–10–27
92–	2 cc.	5–18–28	1,816	4.5	2.0	9.00					199–	4–15–27 (b)
383	daily	6–19–28	1,783	5.9	3.0	17.70	1.77	2.00	1.97		27	6– 9–27

(a) Large open sores.
(b) Shrivelled and discolored spots.

TABLE 15

Procedure XV

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
62–353S	0.5–1 cc. daily	1-31-28 2-21-28		5.0 6.5	2.2 3.5	11.00 22.75	 1.40	 2.07			617	3-22-27 (a) 6 -8-27
62–353S	0.5–1 cc. daily	1-31-28 2-21-28		5.2 6.6	2.1 3.3	10.92 21.78	 1.46	 1.99			H541	(a)
53–343S	2 cc. daily	12-14-27 12-22-27		5.0 5.5	2.5 2.8	12.50 15.40	 1.23				910	6- 6-27 (b) 8-24-27

(a) Thickened yellow regions in skin.
(b) Capon in fair condition.

TABLE 16

Procedure XVI

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
64–355S	1 cc. daily	2-15-28 2-24-28		4.8 5.1	2.1 2.4	10.98 12.24	 1.21				927	6- 6-27 (a) 8-26-27
64–355S	1 cc. daily	2-15-28 2-24-28		5.3 6.4	2.1 3.4	11.13 21.76	 1.95				909	6- 6-27 (a) 8-22-27
84–375S	2 cc. daily	4-14-28 4-24-28		4.0 6.0	1.9 3.3	7.60 19.80	 2.60				10	(b)
84–375S	2 cc. daily	4-14-28 4-24-28		5.0 7.0	2.3 3.2	11.50 22.40	 1.95				912	6- 6-27 (b) 8-25-27

(a) Deep open sores.
(b) Capon in fair condition.

TABLE 17

Procedure XVII

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
63–354S	3 cc. daily	1–31–28 2–10–28		4.5 4.5	2.4 2.4						548	4–15–27 (a) 6– 9–27
63–354S	3 cc. daily	1–31–28 2–10–28		5.4 5.3	1.7 1.7						188	3–22–27 (a) 6– 8–27
65–356S	3 cc. daily	2–14–28 2–24–28		3.7 3.5	1.1 1.0						612	4–15–27 (a) 6– 9–27
100S	3 cc. daily	12–15–27 12–22–27		4.2 4.2	2.0 2.0						904	6– 6–27 (a) 8–26–27

(a) Blue-green shrivelled spots.

TABLE 18

Procedure XVIII

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
54–344	3 cc. daily	12–16–27 12–23–27		4.5 5.3	2.1 2.5	9.45 13.25	 1.40				911	6– 6–27 (a) 8–22–27
54–344	3 cc. daily	12–16–27 12–23–27		4.8 5.7	2.0 3.2	9.60 18.24	 1.90				927	6– 6–27 (b) 8–26–27
55–345	2 cc. daily	12–16–27 12–23–27		4.4 4.6	2.5 2.6						925	6– 6–27 (a) 8–26–27
55–345	2 cc. daily	12–16–27 12–23–27		4.4 4.4	1.8 1.8						906	6– 6–27 (c) 8–22–27

(a) Small open sores.

(b) Skin in good shape.

(c) 12–29–27. Dead. No testis tissue. Much subcutaneous non-resorbed material.

TABLE 19

Procedure XIX

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
45–	2 cc.	12-23-27		5.9	2.1	10.92					930	6- 6-27 (a)
332	daily	1- 3-28		6.9	2.9	18.56	1.70					8-27-27
44–	2 cc.	12-23-27		5.0	2.3	11.5					912	6- 6-27 (b)
331	daily	1- 3-28		4.9	2.2	10.78						8-23-27

(a) Fair condition.
(b) Subcutaneous nodules.

TABLE 20

Procedure XX

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
72–	1-2 cc.	3-13-28		5.0	1.2	6.00					188	3-22-27 (a)
363S	daily	3-27-28		5.5	1.6	8.80	1.47					6- 8-27
72–	1-2 cc.	3-13-28		5.5	2.6	14.30					617	3-22-27 (a)
363S	daily	3-27-28		5.8	2.8	16.24	1.13					6- 8-27
73–	1 cc.	3-13-28		4.3	2.2	9.46					918	6- 6-27 (b)
364S	daily	3-23-28		5.1	2.6	13.26	1.40					8-24-27
73–	1 cc.	3-13-28		5.0	2.4	10.50					904	6- 6-27 (b)
364S	daily	3-23-28		5.6	2.6	14.56	1.39					8-22-27

(a) Skin thickened and yellow. Subcutaneous lumps.
(b) Skin rather yellow.

TABLE 21

Procedure XXI

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
86–	2 cc.	4-24-28		4.5	2.1						41–	4-15-27 (a)
377S	daily	5- 4-28		4.4	2.1						27	6-10-27
86–	2 cc.	4-24-28		4.5	2.0						199–	4-15-27
377S	daily	5- 4-28		4.5	2.0						27	6- 9-27

TABLE 21—*Concluded*

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
87–3788	2 cc. daily	4–24–28 5– 4–28		5.0 6.4	2.4 3.1	12.00 19.84	1.65				608–27	4–15–27 (a) 6– 9–27
87–3788	2 cc. daily	4–24–28 5– 4–28		4.5 5.4	2.1 3.0	9.45 16.20	1.71				347	4–15–27 6–10–27

(a) No skin reaction.

TABLE 22

Procedure XXII

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
66–357	2 cc. daily	2–15–28 2–24–28		5.1 7.0	2.5 3.7	12.75 25.90	2.03				929	6– 6–27 (a) 8–25–27
66–357	2 cc. daily	2–15–28 2–24–28		4.1 6.5	2.0 3.5	8.20 22.75					925	6– 6–27 (b) 8–26–27
67–358	2 cc. daily	2–15–28 2–24–28		4.9 5.6	2.2 2.8	10.78 15.68	1.45				903	6– 6–27 (c) 8–22–27
67–358	2 cc. daily	2–15–28 2–24–28		4.4 5.5	2.4 2.9	10.56 15.95	1.51				905	6– 6–27 (c) 8–22–27

(a) Subcutaneous lumps.
(b) Open sores.
(c) Blue-green spots, then open sores.

TABLE 23

Procedure XXIII

FRACTION		DATES	WEIGHT	COMB MEASUREMENTS							BIRD RECORD	
Number	Amount			L.	H.	(LH)	Index of growth 10d.	20d.	30d.	Fin.	Number	Hatched, Castrated
56–346	2–3 cc. daily	1–20–28 1–31–28		4.4 4.3	2.2 2.1						918	6– 6–27 (a) 8–24–27
56–346	2–3 cc. daily	1–20–28 1–31–28		5.6 5.4	2.4 2.4						624	3–22–27 (a) 6– 8–27
60–3508		1–26–28 2– 5–28		5.3 5.8	2.5 2.8	13.25 16.24	1.22				12	4–15–27 9–15–27

(a) Skin thickened, yellow-blue spots. Capon quite feeble.

Fig. 1. Capon 41–27. Injected daily with 2 cc. of fraction 92–383 from 5–18–28 to 6–11–28 and with 2 cc. of fraction 92–385 from 6–12–28 to 6–19–28.

5–18–28. Photographed just previous to starting injections. Comb length 4.5 cm., height 2.1 cm.

5–29–28. Comb length 6.1 cm., height 3.1 cm. Index of growth 2.00.

6–8–28. Comb length 6.6 cm., height 3.4 cm. Index of growth 2.37.

6–15–28. Comb length 6.3 cm., height 3.4 cm. Index of growth 2.27.

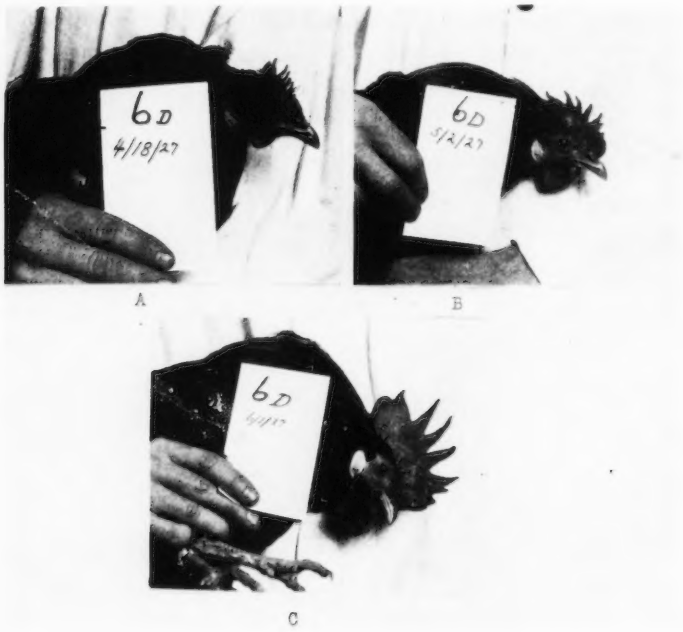

Fig. 2. Capon 6 D, a prepuberally castrated cockerel, received daily injection of 2 cc. of lipoid fraction from bull testicles from April 16, 1927 until May 16, 1927 and received 3 cc. of lipoid fraction thence until June 3, 1927.

Print A: Comb measurements 2.5 by 4.6 cm.

Print B: Comb measurements 4.2 by 7.8 cm.

Print C: Comb measurements 7.0 by 11.7 cm.

DISCUSSION. One of the most interesting assays was on capon 565, the only bird among the large group tested which, in addition to comb growth, evidenced other male characters such as crowing and aggressive behaviour when confronted with hens (tables 3, 11, 4, 10). Comb growth on fraction 42–327 and 42–336 was strong and continuous, the index finally was 5.02. On November 29, 1927, the capon crowed repeatedly and did so again on December 1, 2, 3, 7, 8 and 9. At the latter date the injections were discontinued, the capon was kept under further surveillance but it was not observed to crow again. On December 2 the bird was tested with two hens and showed aggressive behaviour. An exploratory operation was

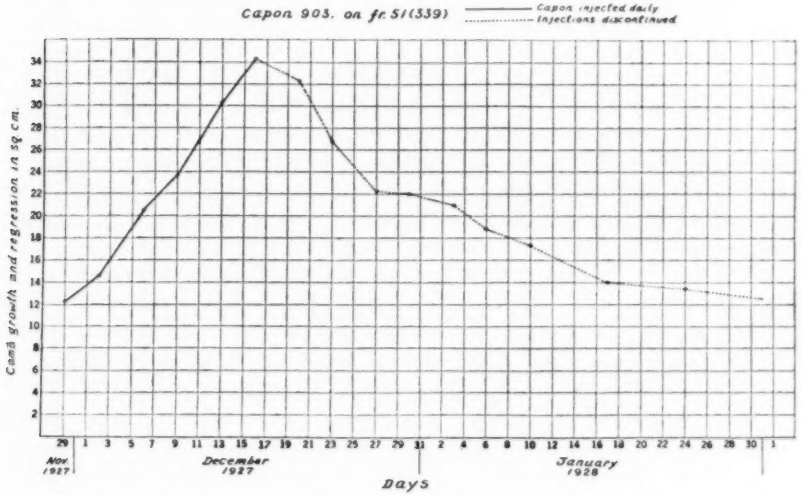

Fig. 3. Graph showing comb growth on capon 903 on fraction 51-339

performed on capon 565 February 22, 1928, and the site of the removed testes was found perfectly clean.

In comparing two birds used in assaying a given fraction it appears that two factors are involved: the potency of the extract and the reactivity of the capon tested. Also, we are dealing with extracts which in addition to their capacity of inducing comb growth in the capon are definitely toxic to the birds in the unfavorable local conditions produced and in some instances in the unfavorable effects caused to the organism as a whole. It might be possible to obtain reactions observed in capon 565 quite generally were the toxic effect divorced from the stimulating one without change of the latter.

From the summarized procedures it is desired that attention be called to several findings. Heating the lipoids in the first alcoholic solution to

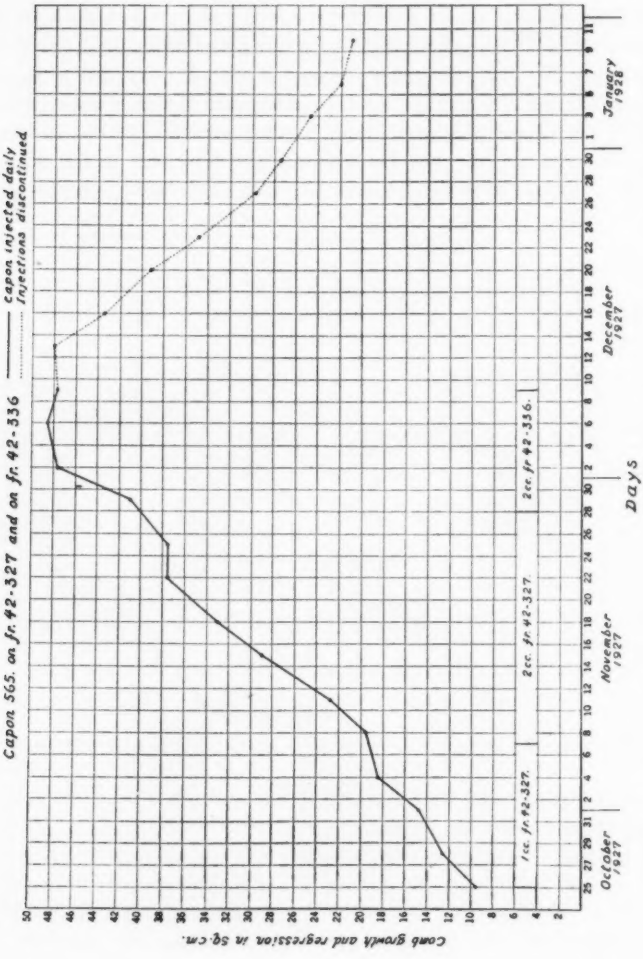

Fig. 4. Graph showing comb growth on capon 565 on fraction 42-327 and 42-336

dryness with slight *charring* on a water bath destroyed activity. Absolute alcohol precipitates much material that is shown to be relatively inactive by our assays. A greater volume of precipitate is obtained at lower temperatures (summarized procedures IV and V) and thus a marked degree of purification with slight loss of activity can be obtained in this manner. With reconcentration of the alcoholic filtrate after the first precipitation and again adding an excess of absolute alcohol at low temperature appreciable activity appears in both the second filtrate and the second precipitate. This is the case in samples under summarized procedure X. Compare the assay of sample 89–380 with that of sample 90–381.

Acetone precipitates from a concentrated benzene solution at a given temperature a greater amount of inactive or slightly active lipoid than does absolute alcohol. Methyl alcohol added in excess to the active lipoids behaves similarly to absolute alcohol.

If the lipoids are placed in a thin layer over filter paper strips and exposed to liquid ammonia the greater part of the activity will, after repeated extractions with fresh portions of the solvent, pass into solution. Cholesterol with other material is left almost quantitatively on the filter strips (18). Some activity is recoverable from the strips by using benzene as a solvent following the ammonia treatment—more particularly if the strips are too heavily coated with the lipoids.

Concentrated hydrochloric acid in procedure IX destroyed the activity of a benzene solution. Benzene stock solution has been found to have no marked loss of activity after five to six months' standing. After nine months the assays seem to show some loss of activity from this stock solution. Destruction of the active lipoid in benzene by exposure to light and oxygen occurs but slowly as indicated in summarized procedure XI. Light seems more a factor than oxygen. More detailed investigation is necessary before positive statements can be made.

Varying the per cent of ethyl alcohol shows interesting results in the purification process as has been found by Doisy with other lipoid mixtures. In summarized procedure XX a preparation having been treated by the cold absolute alcohol precipitation process was fractionated between seventy per cent alcohol and petroleum ether with an approximately equal division of the activity. In summarized procedure XXI a preparation having been treated by the acetone precipitation process was fractionated between eighty per cent alcohol and petroleum ether. By concentrating the alcoholic solution and keeping it at a temperature of zero degree Centigrade, relatively inactive material was precipitated. The filtrate showed fair activity on a capon, dosage of approximately 225 mgm. daily—the best purification thus far obtained without the employment of ammonia as a solvent.

Alkali exposure in saponification for eighteen hours completely destroyed the activity. Some activity was recovered in the non-saponifiable portion

(ether extraction of soaps) after saponification for nine hours only. The employment of alkali in the preparation and purification of active extracts should be pursued farther. It may be possible to remove much inert material without great impairment of activity by rapid saponification processes.

Liquid ammonia treatment after precipitation of many relatively inactive materials by acetone or alcohol yields the most active final preparation obtained at the present. Sample 84–375 serves to illustrate the weight of solids in the filtrates at various steps in the procedure. Aliquot portions are taken at various stages to ascertain the amount of lipoid mass. A given amount of the dissolved lipoid is taken by a 1, 2 or 5 cc. delivery pipette and placed in a tared evaporating dish. After evaporation of the solvent on a steam bath the dish is placed in a vacuum desiccator containing calcium chloride and left for twenty-four hours before weighing.

Two liters of benzene stock solution (s) representing 167.8 grams of solids were concentrated to about 200 cc. To this was added acetone in the amount of 1500 cc. The material was allowed to stand for twenty-four hours and after filtration it was determined that 54.43 grams solids were in the filtrate and 113.3 grams of solids were present in the precipitate. After concentrating the acetone solution to a thin syrupy consistency by distillation under reduced pressure the lipoids were absorbed on strips of filter paper and extracted with liquid ammonia in thermos bottles. All ammonia portions were combined and the solvent allowed to distil away. The ammonia soluble residue was dissolved in benzene—the total amount of solids calculated was 6.74 grams. By using a mixture of alcohol, glycerol and olive oil as a menstruum an emulsion of this material in a total volume of 90 cc. was made. Hence 1.0 cc. of preparation for injection contained 75 mgm. of testicular lipoids. Two cubic centimeters of this, or 150 mgm., daily injected into assay animal gave good growth of the comb—for a ten-day assay indices of 2.6 and 1.9 were obtained.

For maximum growth of the comb of the capon crude lipoids representing at least 150 to 200 grams of testicular tissue (fresh tissue weighed after being ground) are injected daily. For much of the work more than a pound of tissue is represented by the daily dose. The longer the procedure and the more involved the separations of the lipoid the more tissue is represented by the daily capon dose. The optimum amount of alcohol for extraction and the optimum degree of concentration under reduced pressure before fractionation with benzene is not fully determined.

We find that some of the capons included in the series vary from one to two years of age and within these limits no difference in reaction to the injections attributable to age differences could be observed.

It seems clear that repeated injections performed on the same bird in no way cause a diminution of reactivity, that is to say, of the degree of

comb growth obtained, provided, first, that the condition of the bird remains good and, secondly, that it recuperates between treatments. We find a characteristic slowing of the rate of comb growth in prolonged assays about the third week of a series of injections. Often the comb progresses no further after the third week. This is more often noticed with the more purified preparations.

It is noted that some of the crudest preparations injected into capons living in one building have produced better ultimate comb growth than similar fractions assayed on similar capons in other living quarters. This peculiar occurrence is not explained.

Two bilaterally castrated hens, when injected with 2 cc. daily of the active material for six weeks, gave indices of comb growth of 3.4 and 4.8 respectively. Administering the material orally to a capon in doses of 1 cc. twice each day gave negative results.

We have not freed to our complete satisfaction the active preparations of non-absorbable fats which cause inflammation at the site of injection.

We find no justification in these studies for suggesting whether the testicle presents one or several extractable materials affecting secondary sex characters of males.

The same extraction procedure was applied carefully to freshly ground calf thymus. The results of the assays with even larger doses of these lipoids have been uniformly negative as reported previously (1), (2), and as described in this paper. Detailed control studies of the procedure of extraction as applied to many tissues and the consistently negative results from the assay on capons are reported by Gallagher in a paper appearing in THIS JOURNAL. Some sixteen different procedures were followed in the original attempt to obtain a fraction from freshly ground bull testicles, or guinea pig or rat testicles, which would give some indication of the presence of the activity desired. Many of the lipoid separations, saline extracts, acid or alkaline extracts, and glycerol extracts reported in the literature were repeated in the hope of finding a dependable, definite activity affecting consistently and positively some of the secondary sex characters of the brown leghorn. Positive results were found only with the lipoids obtained as described.

In conclusion the authors wish to express their indebtedness to Prof. Fred C. Koch and Prof. Frank R. Lillie for making possible this cooperative work between the Department of Physiological Chemistry and Pharmacology and the Whitman Laboratory for Experimental Zoölogy. Further, the authors wish to acknowledge the thoughtful criticism and supervision received during the course of the work.

SUMMARY

1. A benzene soluble lipin fraction obtained from bulls' testicles when injected into brown leghorn capons causes growth of the comb, wattles and ear lobes.

2. The amount of lipin material injected daily represents from 150 to 500 grams fresh testicle tissue.

3. Although the reactivity of individual capons varies, these variations are of such a nature that this biological reaction can be used as a rough quantitative method for assaying such lipin preparations.

4. Very frequently serious local reactions appear at the site of injection and much unabsorbed material may remain.

5. When injected into bilaterally castrated hens good comb growth was induced.

6. The crude lipin found in the benzene soluble fraction can be freed somewhat from non-potent material by fractional precipitation by methyl alcohol, ethyl alcohol and acetone or by extraction by liquid ammonia.

7. Nine hours' boiling of the active material with alcoholic sodium hydroxide still leaves considerable potency in the non-saponifiable fraction.

8. The activity was lost completely after boiling with alcoholic sodium hydroxide for eighteen hours.

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ON THE EFFECTS OF INJECTING LIPOID EXTRACTS OF BULL TESTES INTO CASTRATED GUINEA PIGS[1]

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In the preceding paper (McGee, Juhn and Domm) a method of extraction and fractionation of testicular lipoids has been described that has yielded material having a marked stimulating action upon the growth of the comb when injected into castrated cocks. It is of especial interest, therefore, to determine whether similar preparations injected into castrated mammals can be shown to have such effects as does the internal secretion, or hormone, of the testicle.

One of the major difficulties confronting investigators in the study of the testicular internal secretions of mammals is the lack of dependable, quickly applied indicators for the hormone. Many different effects of castration of mammals have been recorded, most or all of which might be classed as somatic, metabolic, or psychical. But extensive as are the changes noted for the entire group, it is strikingly evident to workers in this field that few, if any, changes are sufficiently distinct, dependable, and rapid to serve adequately as indicators of the effectiveness of the hormone should this be successfully extracted and introduced into castrated animals.

When animals such as the rat and guinea pig are castrated early in life it is well known that the seminal vesicles, prostate and penis usually remain relatively undeveloped. The variations in size that one notes in a very large series of animals, however, make the detection of submaximal hormone effects very uncertain. The normal variations in size in normal, unoperated animals, as well as in castrated animals not only makes it difficult to properly integrate hormone effects, but the situation is even more complicated when one wishes to test extracts for hormone properties on adult animals; for it is all but impossible to draw a proper balance between results of possible stimulation from an extract and the degeneration from castration. In some cases it has appeared quite clear that testis grafts remaining in a living condition in an animal for many months after

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transplantation stimulated the development of the accessory organs of reproduction beyond what would have been found had no transplantations been made (Steinach, Moore, Sand). In other cases it has been impossible to be certain that conditions actually observed had been produced by a hormone generated in the graft (Moore, 1926). Steinach (1916, 1920) has considered it possible to detect hormone influences from testis grafts by observations on animal size, fat deposition, condition of the hair coat, pelvic changes, relative brightness of the eye, etc. He has not been followed in this belief, however, by many investigators and Moore (1919, 1921, 1922) has criticised such criteria because of their uncertainty and the evident possibilities of varied interpretations.

The psychical nature of the animal has likewise been emphasized by Steinach as an indicator of hormone effectiveness. The interest of the male in the female; the docility under handling; fighting propensities, or lack of them he believes to be important. Variations, again, are too great to have any value. Moore has repeatedly noted the persistence of the copulatory instinct in the male guinea pig for months after castration even when this is done as early as thirty days after birth, and Stone (1927) has shown that the male rat may copulate for eight months after castration.

When one attempts to utilize practically any of the criteria mentioned as a test for the internal secretions of the testis, poor and undependable as they are, usually some months are required for any questionable change to have expressed itself. Hoskins (1925) determined that spontaneous activity, as registered by revolving cages, showed a marked change in the castrated rat, but whether this indicator could be easily employed to test for effectiveness of introduced preparations over fairly long periods of time is unknown to the writers. Hoskins himself (1927) was unable to note any modifying effect of testis extracts prepared by the Allen and Doisy method.

It is obvious, therefore, that progress in isolation of the testis hormone depends to a great extent upon a suitable indicator for hormone effects. Given a dependable indicator for hormone effects the task of extraction and purification of the substance may proceed.

The "spermatozoön-motility" test for the internal secretion of the testis, which has been employed extensively by Moore (1927, 1928a, 1928b) in studying hormone production by the testicle in situ has proven to be a sharp indicator. Having studied the reaction under a variety of conditions, readings having been made upon approximately four hundred cases, an attempt was begun by Moore to study the effect on this reaction of injecting lipoid extracts of sheep testicles into castrated guinea pigs. It was soon learned, however, that McGee had been able to separate fractions of lipoid extracts from bull testes that showed definite effects in promoting comb growth in capons (McGee, 1927). Due to the efforts of Professors F. R. Lillie of the Department of Zoölogy and F. C. Koch of the Depart-

ment of Physiological Chemistry and Pharmacology, a coöperative scheme of attack on these problems was launched in which extractions and refinements of the materials were carried out by McGee in the Department of Physiological Chemistry and Pharmacology and the assay of different preparations by workers in the Department of Zoölogy. The paper preceding this (McGee, Juhn and Domm) has concerned itself with the problems of extraction and refinements and the effects of injection of various preparations of these testicle extracts into castrated cocks. The present paper embodies some of the results obtained from injecting extracts (prepared by McGee) into castrated guinea pigs, the results being based upon observations on the motility of spermatozoa. All injections have been made and the results tabulated by Moore.

This coöperative scheme or the extensiveness of the program under consideration would not have materialized but for the efforts of Professors Lillie and Koch. It is with the utmost satisfaction that we record our great indebtedness to them for their efforts, coöperation and stimulating guidance during the progress of our work.

MATERIALS AND METHODS. The methods of preparation of the extracts will receive but brief mention in this paper. The preceding paper (McGee, Juhn and Domm) deals with the various procedures employed in many efforts at refinement, and a discussion of the literature in regard to the biochemical methods previously employed.

The following general procedure of extraction applies to all materials in the first stage of preparation and a few more specific details will be given for each sample upon which an assay was attempted. The testicles of bulls were obtaind from Swift & Co. and Armour & Co. of Chicago; they were finely ground and to the mass were added approximately three volumes of 95 per cent alcohol. After standing for three to ten days the material was filtered through cheese cloth and the tissue discarded. The alcoholic filtrate was concentrated under reduced pressure, on the water bath at 90°C., to approximately one-eighteenth its original volume. This alcoholic sludge was shaken with an equal volume of benzene. The supernatant benzene was withdrawn and served as a benzene stock solution. Portions of this stock were treated in a variety of ways as will be noted in appropriate places following.

The spermatozoön-motility test has been adequately discussed elsewhere (Moore, 1927, 1928a, 1928b) as it has been employed under experiment; its essentials depend upon the relative lengths of time spermatozoa remain alive in the epididymis and exhibit powers of movement upon proper stimulation. Thus, when the two testes of the guinea pig have been removed, but the epididymides (tail portion only) containing millions of spermatozoa remain in the scrotal position, the spermatozoa remain alive and capable of exhibiting movement when suspended in physiological

saline solutions up to twenty-three days after castration. If one testis only is removed, the corresponding isolated epididymis will contain spermatozoa capable of exhibiting motility for a period of sixty-five days. The hormone produced by the opposite intact testicle is responsible for increasing this length of life, as exhibited by the capacity for motility, from twenty-three to sixty-five days. Small living portions of a testis increase the spermatozoön life very materially, and aspermatogenetic testicles are as effective in prolonging sperm life as are normal testes (Moore, 1928b).

In attempting an assay of the extracted lipoids of the bull testicle, the two testes of a guinea pig are removed and the isolated epididymides, with their spermatozoön content, carefully replaced in the scrotum. This latter precaution is necessary since the thermoregulatory function of the scrotum is necessary for continuance of sperm life (Heller, 1928).

The materials under test are injected hypodermically and the animals sacrificed at varying intervals. The epididymides are finely hashed with scissors in a small quantity of physiological saline and observed at once under the microscope for spermatozoön motility. To express graded differences in vigor of motility, symbols have been employed, **** to designate the motility of spermatozoa recovered from a normal epididymis, * to denote the weakest movement observed, and 0 the absence of any sperm movement; ** and *** represent intermediate grades of activity. It should be emphasized that quantities of spermatozoa which to all appearances are morphologically normal may be recovered from an epididymis and yet show no movement when suspended in saline. The powers of motility are lost considerably earlier than disintegration changes can be observed.

In actual practice, therefore, materials are injected into castrated animals whose epididymides have been isolated from their respective testes. Despite the fact that spermatozoa have never been observed to show motility for periods longer than twenty-three days after removal of the testes, an arbitrary choice of thirty days after castration has been accepted as the minimum amount of time for the capacity for motility to persist in order that a result may be accepted as a positive one.

EXPERIMENTAL. I. *Sample 39–324* (McGee's numbers) was prepared as follows:

A portion of the benzene stock solution (mentioned above) was concentrated to a small volume and seven to eight volumes of cold acetone added. After allowing the material to stand in a freezing room for six to eight hours the acetone solution was filtered, concentrated in a partial vacuum, and two portions of absolute alcohol added (400 cc. in all) during distillation to aid in removing the acetone. To the final syrupy lipoid mass were added three volumes of olive oil. In this form sample 39–324 was injected into the test animals (guinea pig).

A. October 10–13, 1927. Bilateral epididymal isolation. From table 1 it can be seen that of the three animals injected with 1 cc. of the olive oil solution, spermatozoa from the isolated epididymides retained their capacity to exhibit motility on proper stimulation on the 30th, 35th and 40th day after castration. Those injected with 2 cc. and 3 cc. of the solution showed motile sperm on the 40th day when the spermatozoa were suspended in normal saline solution. Since spermatozoa in isolated epididymides in animals without testes retain their capacity for motility for but twenty-three days, and since such a retention of the capacity for motility for thirty days or longer is considered to be positive evidence of the effect of the testis hormone, it becomes apparent that of the nine test animals, eight showed a positive effect from the injections. The ninth animal (no. 426) observed on the 40th day, showed many normal appear-

TABLE 1

Sample 39–324 (assay)

	ANIMAL NUMBER								
	424	427	430	425	428	431	426	429	432
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
October 20, 1927, injection	1	1	1	2	2	2	3	3	3
October 22, 1927	1	1	1	2	2	2	3	3	3
October 24, 1927	1	1	1	2	2	2	3	3	3
November 3, 1927	1	1	1	2	2	2	3	3	3
Observation on days after operation	30*	35**	40*	40*	40*	40*	40–0	40*	40**

ing spermatozoa, but none were motile. Should it have been observed on the 35th day it likewise would probably have shown motile spermatozoa.

B. A second group of three animals was chosen for an assay on the same sample as the above (sample 39–324) using smaller dosages and injecting more often. Thus animals 466, 467, 468 were subjected to epididymal isolation and injected with 0.5 cc. of the olive oil solution on the 3rd, 5th, 8th, 11th, 15th, 19th, 22nd, 25th, 29th and 33rd day after testis removal.

The results of this procedure were as follows: animal 466, killed on the 46th day after testis removal did not show motile sperm; animal 467 killed on the 47th day had many spermatozoa, a small percentage of which showed motility; animal 468, killed on the 50th day was without motile spermatozoa though many normal appearing ones were present.

The results with sample 39–324 show that a considerable amount of some substance having an effect similar to the testicular hormone was presçnt. Seven animals showed a positive reaction (motile spermatozoa) on the 40th day and one was positive on the 47th day.

II. *Sample 48–335* (preparation). To the benzene stock solution concentrated to a small volume were added four to five volumes of acetone and the solution allowed to stand in a freezing room for twelve hours. Discarding the precipitate, the acetone filtrate was concentrated under reduced pressure to a syrupy consistency which was taken up on strips of filter paper, giving a thin lipoid coat to the strips. After drying in vacuo, on a water bath, for eight hours the strips were placed in thermos bottles and extracted for one week with liquid ammonia. After distilling the ammonia, the ammonia soluble substances were dissolved in absolute alcohol and this was concentrated to a small volume. At the time of injection the concentrated alcoholic solution was added to physiological saline and the alcoholic-lipoid-saline emulsion introduced subcutaneously with a hypodermic syringe. Each dosage of 1 cc. (1 part alcohol solution to 3 parts of saline) was the equivalent of 0.25 cc. of the alcohol-lipoid-solution.

Sample 48–335 (assay). Two groups of three animals each were subjected to epididymal isolation on November 17, 1927. Into animals of one group (animals 460–461–462) was injected 1 cc. of the alcohol-saline emulsion, while into the other group (animals 463–464–465) were injected 2 cc. subcutaneously. Injections into each group were made on the 4th, 6th, 9th, 12th, 16th, 20th, 23rd, 26th, 30th and 34th day after epididymal isolation.

Observations were as follows: animal 461, killed on the 34th day had living spermatozoa; animal 460 on the 47th day showed living sperm; animal 462 on the 51st day possessed spermatozoa but none exhibited motility; animal 463 sacrificed on the 47th day possessed spermatozoa, motile when suspended in saline; animal 464 died on the 46th day and was not observed; animal 465 sacrificed on the 51st day did not have living spermatozoa (latter three received 2 cc. injections).

Again there is a definite prolongation of the capacity for motility of spermatozoa after the injection of the preparations since motile spermatozoa were obtained up to the 47th day after operation. But the effects from the 1 cc. injections were apparently as great as those from the 2 cc. injections.

It is also apparent that the preparations in alcoholic solutions and injected as an emulsion with saline are of approximately the same strength as those tested in a suspension of olive oil.

III. *Samples 58–348 and 59–349* (preparation). These two samples of material, one an extract of bull brain and the other an extract of bull testicles, were prepared similarly. Each started from a benzene stock solution. The benzene stock solution was concentrated to a small volume and to this were added three to four volumes of absolute alcohol; after standing for two days in a cold chamber the inactive lipoid precipitate was

discarded and the filtrate concentrated. The syrupy lipoid mass was taken up on filter paper strips. After extraction with two samples of liquid ammonia, and removing the latter by distillation, the ammonia soluble residue was dissolved in absolute alcohol, which solution was concentrated to approximately 20 cc. This alcoholic solution of lipoid material was added to physiological saline in proportions of 1 to 3 (at time of injection). The dosage employed for injection was 1 cc. per animal, or 0.25 cc. of the alcoholic solution.

Samples 58–348 and 59–349 (unknown samples) (assay). On January 10, 1928, two samples in alcoholic solution were supplied by Doctor McGee under number only, both having been prepared alike and of equivalent concentrations in lipoid content. On January 17th two groups of four animals each were subjected to epididymal isolation and 1 cc. of alcoholic saline emulsion was injected into each animal on the 3rd, 6th, 8th, 10th, 13th, 15th, 17th, 20th, 22nd and 24th day after operation.

On the 30th day after operation all animals receiving injections of sample 58–348 (animals 494–495, 497, 498) were killed and none showed any motile spermatozoa from the epididymides when these were examined in saline solution. Of those receiving the injections of sample 59–349 animal 489, killed on the 30th day, showed motile spermatozoa; animal 493 was dead on the 35th day and no record taken; animal 490, killed on the 35th day, had developed adhesions and scrotal thickening and did not have living sperm; animal 492, killed on the 35th day, possessed millions of spermatozoa, many of which showed vigorous motility when suspended in physiological saline.

Since the finding of motile spermatozoa on the 30th or succeeding day after operation is an indication of a positive effect of the injection, sample 58–348 was reported negative and sample 59–349 was reported positive. Reference to Doctor McGee's records showed the former sample to have been prepared from bull brain and the latter to have been prepared from bull testicle.

IV. *Sample 80–371* (preparation). Three liters of benzene stock solution, representing 251.7 grams of solid were concentrated to approximately 300 to 250 cc. Two liters of acetone were added and the material kept at zero degrees Centigrade for 18 to 20 hours. After filtration 58.4 grams of solids were represented by the filtrate and 193.3 grams of solids were removed in the precipitate. The precipitate thus obtained was not effective in producing comb growth even when as much as one gram per day was injected into a capon (McGee, Juhn and Domm); the material was discarded. The acetone filtrate was concentrated to a syrupy consistency and absorbed on filter paper strips. These were extracted with four fresh samples of liquid ammonia and the extracts combined. The ammonia soluble residue was taken up in benzene and prepared in an alcoholic

solution having a total volume of 62 cc. containing 6.900 grams of solids (1.0 cc. is equivalent to 111.3 mgm). Thus we use for assay less than 2.7 per cent of the lipoids present in the benzene stock solution. It is probable this amount of lipoid does not represent all the active principle of the original material but speculations at the present stage are entirely unprofitable.

Sample 80–371 (assay). Since the lipoid fraction dissolved either in olive oil or in absolute alcohol had proven to exert a positive effect on injection and therefore contained a substance acting to maintain the spermatozoön life as does the internal secretion of the testis, and since the local reaction at the site of the injection was less, and the ease of injecting greater, with the alcoholic preparations, an attempt was made to see how strong a reaction could be obtained with such preparations. It is to be kept in mind that the spermatozoön-motility test indicates positive effects if spermatozoa in the epididymis 30 days after operation preserve the capacity for exhibiting motility. Furthermore, it should be remembered that in a unilateral isolated epididymis in an animal with the opposite testicle normal, and therefore continually supplying the hormone, spermatozoa remain alive and capable of exhibiting motility for 60 to 65 days after preparation of the test epididymis. An attempt therefore was made to introduce the extracted substance into the animal more constantly than in previous assays by injecting more frequently.

For this assay two dilutions were employed; in the one group of six animals 1 cc. of alcoholic-saline emulsion was injected so that each injection represented one-fifteenth cc. of the alcoholic preparation (7.4 mgm.). The second group, consisting of eight animals was injected similarly though with a dilution giving double the amount of the original alcoholic solution or one-eighth cc. per injection (13.9 mgm. lipoid). The first injection was made on the 3rd day following removal of both testes from the epididymides and continued thereafter up to the 52nd day at the rate of approximately five injections per week. Thirty-six subcutaneous injections were given each animal in the course of the experiment with the exception of animal 537 which was killed on the 48th day after thirty-three injections.

Results. Since the maximum reaction was sought, the first observation was made on animal 537 on the 48th day after testis removal. The epididymides of this animal contained quantities of spermatozoa and many of them exhibited a fair degree of movement. This animal had received thirty-three injections of the weaker dose. All other animals of the experiment, thirteen in number, were not observed until the 54th day after operation, during which interval each had received thirty-six injections. In practically all animals many spermatozoa were present in the epididymides but only one (no. 541) revealed any motile sperm when

they were suspended in saline solution. It follows, therefore, that the active substance in the sample was of sufficient strength and introduced sufficiently often to give a positive reading by the spermatozoön-motility test up to 54 days after testis removal. When this result is compared with the findings from animals in which one normal testis was present and continually producing the hormone, and which suffices to preserve the capacity for motility up to approximately sixty-five days, it is realized that the material introduced by injection contained an appreciable amount of a substance in the lipoid portion of extracts of the testicle that had an influence upon the life of the spermatozoa within an isolated epididymis similar to, and almost as strong as the influence of the hormone produced by a normal intact testicle.

Discussion. The problems more immediately concerned with the extraction and purification of the internal secretion of the testicle as well as the effects of the injection of lipoid fractions from the bull's testicle on castrated cocks, are presented in greater detail in the preceding paper (McGee, Juhn and Domm, This Journal and number).

Perhaps one of the greatest difficulties in studying the effect of preparations on the castrated mammal is the hitherto lack of dependable indicators for the substances which are sought for isolation. The spermatozoön-motility test to some degree eliminates this impediment to progress though it is freely admitted that the test is not all that could be desired. Its specificity is considered to be satisfactory but the application of the test is somewhat restricted and at times uncertain. For example, one is unable to make more than one test upon the same animal; after the operation for epididymal isolation and its scrotal return, adhesions often develop with the tunica vaginalis or seminal vesicles which of themselves prevent a correct reading of the reaction, due probably to an atypical scrotal relationship of the epididymis (Heller, 1928); the test can be employed only to show the maintenance of the existing hormone level at the time of testis removal, and it is useless in attempts to test for the restoration of the hormone level once this has completely receded. We are constantly trying new tests for the hormone effect but up to the present we regard the spermatozoön-motility test the best short time test that has yet been employed on mammals.

Despite its restricted application, however, we have obtained very definite results from injecting the lipoid extracts of testicles into castrated guinea pigs. Four lipoid fractions of the bull's testicle varying slightly in their method of preparation, have proven to be effective in prolonging the life of the spermatozoa in isolated epididymides for periods longer than the persistence of the capacity for motility after removal of the testes. Since 23 days is the longest period for this persistence of the capacity for motility in the guinea pig without some manipulative procedure with

testis material, the minimum period of 30 days for a positive effect to be registered is believed to be very conservative. After four injections of preparation 39–324 (olive oil suspension from which the material is believed to be absorbed less rapidly than in emulsions) the persistence of the capacity for motility was retained for 40 days; whereas, after ten injections of the same material the time was increased to 47 days. With emulsions (Prep. 80–371) (36 injections) the time of persistence of the capacity for motility was increased to 54 days. When it is recalled that the indicator depends upon the persistence of life of an isolated cell whose existence is limited to approximately 65 days when the full hormone complement is being provided by two normal, intact testes, an extension from the basic 23 days (when no hormone is being supplied) to 54 days must be considered a strongly positive effect. Previous experiments with this test (Moore, 1928b) have shown that the hormone produced by the intact testis is not stored within the body for any appreciable length of time and the chances are that, like the extracts of follicular fluid, it is lost rather rapidly. It is not surprising, therefore, that the strongest reaction obtained falls a little short of that obtained when the normal testis is continually supplying the substance.

The local reaction to the injection of the preparations has in some cases been rather severe. A local inflammatory reaction to the injected substance produces nodules that on some occasions have broken through the skin and discharged a small amount of pus. Very often frequent injections for a ten day period are accompanied by a loss in weight of from 5 to 20 per cent. Following this initial loss, even when five injections are made in seven days, the animals usually hold their own and many times gain back a considerable amount of the lost weight so that at the end of a month of the continued injection, their initial weight may have been regained. It seems probable that could the animals withstand sufficiently frequent injections one would be able to obtain a persistence of the capacity for motility for as long as would be possible with an intact and normal testicle. We are looking forward to obtaining sufficiently purified products of the extractions that will permit of more frequent injections.

The question may be raised whether the preparations contain the testis hormone. In the first place we do not know whether the so-called internal secretion of the testicle or hormone is composed of one or more substances. Secondly, regarding the specificity of the test employed, manipulations of the testis through castration, cryptorchidism, transplantation, fragmentation, etc., have shown the sperm-motility test to be an indicator of the presence and activity of the testis tissue. It has not been proven that this is the only substance or substances that has the capacity to influence the life of the spermatozoa, but it can be regarded as highly probable that the test is specific. Introduction of testis material through

transplantation that undergoes subsequent autolysis and degeneration has little or no effect upon it, and lipoids from the bull brain, prepared similarly to the testis extracts and injected in the same amounts, and with the same frequency have proven to be without effect on the test; spermatozoa were not capable of showing motility at 30 days after testis removal and injection of lipoids from the brain.

The results here reported can be regarded as positive but must be considered as merely preliminary, in so far as the problem is concerned; our work is continuing rapidly on its extension.

SUMMARY AND CONCLUSIONS

Lipoid extracts of the bull testicle have been prepared which, when injected subcutaneously into guinea pigs whose testes had been removed (epididymides remained in the scrotum), proved to act as do hormones produced by the intact living testicle.

The test employed to denote effectiveness was the spermatozoön-motility test which depends upon extending the life of the spermatozoön in the isolated epididymis. Spermatozoa retain their capacity to show motility on proper stimulation for but 23 days when no hormone is present. After injection of the testis extracts this period has been extended to 35, 40, 47 and 54 days. The limit of this persistence when the normal testicle is present and active is 65 days.

The substances introduced by injection, therefore, must be considered as having a similar effect to the intact testis and for this reason we believe that it contains an active principle of the internal secretion.

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DISTRIBUTION OF TESTICULAR COMB GROWTH STIMULATING PRINCIPLE IN TISSUES

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Earlier work from this laboratory (1) and more recent extensions of this preliminary report (2), (3) have shown that extracts of testicular substance may be obtained which exert a remarkable effect on the secondary sex characters of the capon. More recently these observations have been extended to the mammal (4).

All previous work had been with testicular tissue and a few observations on thymus as a control extract. The following work was undertaken to investigate more thoroughly the specificity of testicular extracts and to determine the extent of distribution of such activity in other tissues.

Extracts of various tissues were made following the routine method of McGee as follows: a given weight of tissue was trimmed of extraneous tissue, ground and extracted with two and one-half volumes of ninety-five per cent alcohol for five days. At the end of this time the fluid was pressed through cloth, filtered and concentrated to a watery sludge under diminished pressure. The sludge was shaken with benzene and the benzene layer siphoned off. The benzene solution was concentrated to a thick syrup under diminished pressure, care being taken to remove traces of benzene as thoroughly as possible. This material was then dissolved in olive oil and used for injection. It was not considered desirable to fractionate the material further since the active principle is easily detected in the benzene soluble material from testicular tissue and since loss by subsequent treatment could be obviated.

The method of assay was that of the comb growth stimulation in capons, this method having been found to give most dependable results. Every detail of the method of preparation and assay was identical with the methods used in preparing and assaying active extracts of testicular substance.

Table 1 contains the results of our extractions.

In all these assays at least twice the quantity of lipin from which a positive result is obtained, using testicular tissue, was injected. The

TABLE 1

TISSUE EXTRACTED	NUMBER OF TRIALS	RESULT OF ASSAY
Bull brain	2	Negative
Beef ovary	3	Negative
Bull pancreas	1	Negative
Bull blood	1	Negative
Bull prostate	2	Negative
Bull thyroid	1	Negative
Bull suprarenal (cortex and medulla)	1	Negative
Bull kidney	1	Negative
Bull liver	1	Negative
Bull seminal vesicles	2	Negative
Bull epididymis	4	3 Positive 1 Negative*

* This negative result we ascribe to the fact that not sufficient material was injected.

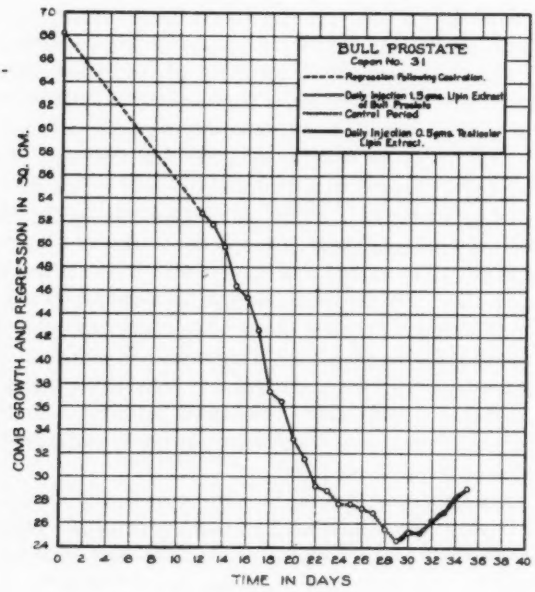

Fig. 1

amount of lipin injected was always over 2 grams per day and half this amount of testicular lipin consistently gives a rapid comb growth.

It is significant that such activity may be extracted from the epididymis and while it does not mitigate the specificity of the testicular extract, it may lead to a change in our views regarding the source of such activity in the testicle. From a few preliminary studies we have the indication that it is present in lesser amount in the epididymis. It likewise follows the same solubility fractionation as testicular extract. We are extending this work further, both as regards its quantitative relationship to testicular extract and its further purification.

The other results in table 1 are self-explanatory. There was never the slightest indication of comb growth. Figure 1 shows an attempt to inhibit the regression of a cockerel's comb following castration by the injection of lipoid extract of prostate. If present in minimal amount the active principle would slow regression so that it might be detected by plotting on a graph. It had no such effect and may be contrasted with the immediate growth following injection of a smaller quantity of testicular extract.

SUMMARY

1. The active principle of testicular substance is not obtained from any other bull tissue except epididymis.

2. The indication is that the active comb growth stimulating principle is not present in as great amount in the epidymis as in the testicle.

3. It is not found in whole beef ovary (follicles intact).

The author wishes to express his appreciation to Prof. F. C. Koch for his kind advice and assistance.

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AN IMPROVED METHOD FOR THE DETERMINATION OF CARDIAC OUTPUT IN MAN BY MEANS OF ETHYL IODIDE

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Three years ago we undertook to use the ethyl iodide method of determining cardiac output in man as devised by Henderson and Haggard (1925). They very kindly instructed us in the technique elaborated by them and we hoped to add our efforts to theirs in the study of some of the many important questions which the new method gave promise of elucidating. It will be recalled that the chief advantage claimed for the Henderson and Haggard method in comparison with the more difficult one of Krogh and Lindhard, or with those depending upon the use of the Fick principle in calculating cardiac output from arterial and venous content of carbon dioxide and oxygen, was based upon the belief that ethyl iodide was rapidly and completely hydrolyzed after diffusion into the blood of the pulmonary circuit; hence that simple estimation of the amount of ethyl iodide which disappeared from inspired air containing it, combined with knowledge of the alveolar concentration of ethyl iodide and its coefficient of distribution between air and blood should give a ready means of calculating the volume of blood which flowed through the lungs during the time of the experimental test.

Experience during the first year of our work was discouraging. In our hands the iodine pentoxide method of analysis of air containing ethyliodide proved to be unreliable. We therefore elaborated an alternate method which, while more laborious, gave accurate results in a series of 20 estimations of weighed quantities. Using this method and a technique extending it to the estimation of ethyl iodide in blood, we discovered that we were unable to confirm the determinations of Henderson and Haggard of the coefficients of distribution between air and blood, nor were we able to confirm their finding that ethyl iodide is so rapidly destroyed in blood that its passage to the venous side of the circulation is negligible (Starr and Gamble, 1927).

Consequently we were confronted with the necessity of abandoning the original project altogether or else of so altering the plan of the method that it should yield results in which we could have confidence. The latter alternative was chosen. From the work to be described in this paper it has

become apparent that ethyl iodide in conjunction with the Fick principle may be used for consistent and, we believe, reliable calculations of cardiac output in man. In process of development new estimations of the coefficient of distribution between air and blood have been made. Experiments have been performed on anesthetized dogs and normal men inhaling ethyl iodide which demonstrate that the ethyl iodide content of arterial blood may be accurately estimated from that of alveolar air. Other series of experiments demonstrate that in dogs inhaling ethyl iodide for several minutes the ethyl iodide content of mixed venous blood does not change materially during rebreathing and may be estimated from the content in the rebreathed air. Additional evidence, secured in experiments on men, indicates that the ethyl iodide content of mixed venous blood of men may likewise be estimated from analysis of rebreathed air. Finally the new method has been tested in a number of experiments by applying it to estimations of flow through dogs' lungs, perfused at known rate, with satisfactory agreement of results.

During the course of the work it was found desirable to introduce further refinements into the technical procedures described in our previous communication. These changes are detailed in the first section of this paper. Experiments were also made to test further points at issue among those who are interested in the Henderson and Haggard method. A statement of these points and of our own evidence bearing upon them is presented in the communication immediately following this.

TECHNICAL PROCEDURES. The method previously described for the estimation of ethyl iodide in air (precipitation as silver iodide after decomposition by nitric acid and titration of excess silver nitrate) has not been essentially changed. When samples of more than 500 cc. are analyzed 40 hours are allowed to elapse to insure completeness of reaction.

In the distillation of blood preliminary to the determination of its ethyl iodide content, the prevention of foaming by injecting capryl alcohol before the blood has permitted us to reduce the pressure to 20 mm. Hg or less. Samples are distilled promptly to avoid loss from the slow destruction of ethyl iodide.

In our previous work with blood, the largest samples with which our method was suited to deal were 5 cc. This fact necessitated the use of concentrations of ethyl iodide in the determination of distribution coefficients, and in experiments on dogs, very considerably higher than would be encountered in the blood of man during an ethyl iodide experiment. To meet possible criticism on this ground the method for blood analysis has been modified so that as much as 60 cc. of blood can be subjected to distillation and its ethyl iodide collected and measured. This, in turn, permitted us to reduce the concentrations of ethyl iodide used in estimations

of distribution coefficients to the range of those which are encountered in work upon man, and to determine the content of arterial and venous blood of man during a blood flow estimation. The modified method which we use for samples of 10 to 60 cc. is as follows:

Method for the estimation of minute amounts of ethyl iodide in 60 cc. of blood. Volatilizing tubes (Starr and Gamble, 1927, fig. 1) of 200 cc. capacity have been constructed, the neck bent at an angle of 135° at a point close to the collecting tube. After evacuation to 20 mm. Hg or less, 0.2 cc. of nonyl alcohol is injected, followed by the blood sample. The end of the volatilizing tube containing the blood is immersed in water at 75°C. until approximately 5 cc. of fluid have distilled into the collecting tube. Air is now admitted through the needle valve until, on replacement

TABLE 1

Recovery of known amounts of ethyl iodide from 50 cc. of blood

DATE	ETHYL IODIDE		ERROR	
	Added	Recovered		
	mgm.	*mgm.*	*mgm.*	*per cent*
November 15	0.52	0.52	0	0
	0.93	0.86	−0.07	−7.5
November 21	0.91	0.86	−0.05	−5.5
	1.76	1.68	−0.08	−4.5
November 25	0.49	0.49	0	0
	1.24	1.27	+0.03	+2.4
December 6	0	0.02	+0.02	

Mean difference = −0.02 mgm.
Average deviation from mean difference = ±0.035 mgm.

in the water, boiling no longer occurs. The hot water is then allowed to enter through this valve until the volatilizing tube is filled and all gas displaced into the collecting tube. The collecting tube is now detached from the volatilizing tube, and the analysis completed as previously described.

Seven consecutive estimations of known amounts of ethyl iodide added to 50 cc. of blood by the method previously described (Starr and Gamble, 1927) are shown in table 1. The error is of the same order of magnitude as when 5 cc. of blood are employed.[1]

[1] During the course of the investigation numerous control analyses were performed, with the following results:

I. Additional estimations of known amounts of ethyl iodide added to 5 cc. of blood after dilution with water as described (Starr and Gamble, 1927).

Collection of air containing ethyl iodide. The method previously described, i.e., rapid displacement of distilled water in an ordinary sampling tube was usually employed. But in working with dogs it was more convenient to collect alveolar air in a 100 cc. glass syringe, where it was measured before transfer to an evacuated sampling tube. Some of the samples of rebreathed air of dogs were collected in the same way, but the majority were trapped in a peritoneal membrane bag ("fish skin" condom) and drawn into a smaller sampling tube of known capacity. As there is a slow loss of ethyl iodide in such a bag, this transfer was made promptly. Volume measurements were made at room temperature and analytical results were corrected to 37°C. and saturation with water vapor. Control experiments proved the accuracy of these procedures within the analytical error.

IMPROVED ESTIMATION OF THE DISTRIBUTION COEFFICIENT OF ETHYL IODIDE BETWEEN AIR AND BLOOD IN VITRO; ITS CONSTANCY AT DIFFERENT CONCENTRATIONS AND IN NORMAL PERSONS, ITS VARIATION WITH THE TEMPERATURE AND IN ANEMIA. The technique previously described (Starr and Gamble, 1927) has been somewhat modified. To prevent evaporation and change in water vapor saturation during equilibration the air is saturated with water before entering the tonometer or sampling tube. Twelve cubic centimeters of blood are introduced into a 250 cc. tonometer, and a current of air containing ethyl iodide (4 to 5 mgm. per 100 cc.) passed through at the rate of 1 liter per minute. Before entering the tonometer it passed through a glass spiral, and a small wash bottle containing distilled water. A test showed that it became 99 per cent saturated. These several pieces of the apparatus were immersed in a water bath, kept at 37°C. During the passage of the air, the tonometer is rotated slowly back and forth and at the end of 30 minutes or more 5 cc. samples of blood are taken for analysis. In the calculations allowance is made for the measured dead space of the syringe, filled with distilled water.

Mgm. added	1.66	0.63	0.88	0.98
Mgm. recovered	1.70	0.59	0.96	1.00

II. Known amounts added to 5 cc. of 5 per cent gum acacia in 0.9 per cent NaCl by the same technique:

Mgm. added	1.20	1.89	1.51
Mgm. recovered	1.08	1.83	1.46

III. Under certain conditions the full amount of ethyl iodide thus added to blood cannot be recovered. As this difficulty occurred in summer, we considered that increased hydrolysis at the higher room temperature might be a factor, and succeeded in recovering the full amount by cooling the ethyl iodide, water, and blood in an ice bath prior to distillation. The loss at room temperature did not occur with a new lot of ethyl iodide tested in early autumn and had not occurred with another lot during the previous summer. Consecutive estimations of known amounts added to 5 cc. human or dog blood in summer by dilution in the cold follow:

Mgm. added	2.56	3.92	2.94	2.61	0.97	0.85	0.83	0.79
Mgm. recovered	2.40	4.03	2.66	2.80	0.94	0.84	0.75	0.75

TABLE 2

Coefficients of distribution of ethyl iodide between air and human blood

DATE	SUBJECT	O_2 CAPACITY	ETHYL IODIDE				DISTRIBUTION COEFFICIENT	REMARKS
			In air		In blood			
			Before equilibration	After equilibration	First sample	Second sample		
		volumes per cent	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*		
November 30.....	S.		0.16	0.17	0.98		6.0	500 cc. air. 50 cc. blood analyzed
November 30.....	S.		3.82	3.63	22.6	21.0	6.3	Red blood cells 4,480,000
January 20.....	S.	20.85	3.65	3.61	22.3		6.1	Hemoglobin 93 per cent
March 8.....	S.		4.56	4.57	30.7	29.1	6.6	Fasting
December 7.....	G.		0.13	0.12	0.81		6.4	500 cc. air. 50 cc. blood analyzed
December 7.....	G.		3.67	3.73	24.0	24.7	6.6	Red blood cells 4,800,000
January 25.....	G.		4.64	4.54	28.5	32.8	6.7	Hemoglobin 94 per cent
March 14.....	G.		4.52	4.53	27.8		6.1	
January 27.....	W.		4.47	4.53	25.9	26.5	5.8	
January 31.....	H.	21.4	4.37	4.54	23.6	27.8	5.9	
February 7.....	T.	23.6	4.78	4.79	31.1	31.8	6.5	
February 9.....	C. S.	22.1	4.57	4.80	27.3	25.2	5.6	
February 14.....	S. T.		4.60	4.60	26.1	29.0	6.0	Equilibrated at 37°C.
February 14.....	S. T.		4.40	4.26	22.1	21.9	5.1	Equilibrated at 40°C.
November 10.....	Sim.*	13.65	3.87	4.14	21.0	21.8	5.4	Anemic patient
November 7.....	Sim.*		1.97	1.95	9.61	10.45	5.2	Red blood cells 4,030,000
November 11.....	Sim.*		1.78	1.86	9.50	8.64	5.0	Hemoglobin 60 per cent
November 9.....	Sim.*		0.28		1.4		5.1	

Average coefficient of seven normal subjects at 37°C. = 6.07
Average deviation ±0.28
Average of thirteen determinations on seven normal subjects at 37°C. = 6.18.

* Four determinations made on a single sample. Determinations made on the same day were performed on a single sample.

This modification has been used for all determinations of the distribution coefficient of human blood and for all dogs' blood since March, 1927. In two experiments, to perform the equilibration with the concentrations of ethyl iodide found in alveolar air during a determination of blood flow, 55 cc. of blood were equilibrated in a tonometer of 1200 cc. capacity, with air containing 1.2 to 1.7 mgm. per liter, a single sample of 50 cc. blood being analyzed.

The results on human blood are recorded in table 2. Repeated determinations on the same blood equilibrated at different ethyl iodide concentrations (down to that of alveolar air) and on blood from the same subject on different days, agree within the experimental error. Each of the two series on S and G shows an average variation from the mean of 0.2, an amount which approximates that caused by the probable analytical error.

The distribution coefficients for the blood of seven normal subjects at 37° average 6.1, varying from 5.6 to 6.5. This is distinctly lower than the figure of 7.6 that we reported previously (Starr and Gamble, 1927). An obvious difference between the two series is that in the former, the size of the blood samples was usually under 2 cc., never over 3 cc. In the present series greater experience permitted the use of samples of 5 cc. and over, the errors of analysis and of measurement of blood being reduced thereby. The error from evaporation of water from blood during equilibration is absent from our present series. Therefore we regard our previous results as superseded. That our recent results have been confirmed in vivo will be seen below.

Though this series of distribution coefficients on human bloods is not large, it suggests that they do not differ greatly in normal individuals. The average deviation from the mean, amounting to 0.28, is only slightly greater than the deviation in repeated determinations on the blood of the same subject and than the effect of the probable analytical error. The value of 5.2 found in an anemic patient, and the value of 5.1 at 40° in blood which gave a coefficient of 6.0 at 37° indicate that the average normal coefficient cannot be used in all persons, or in febrile conditions.

The distribution coefficient of human blood is significantly less than that of dogs. Blood from eight dogs equilibrated at 37° gave an average value of 10.7, the individual coefficients varying from 9.5 to 11.8.

EXPERIMENTS ON DOGS AND MEN INHALING ETHYL IODIDE, DEMONSTRATING THAT THE CONTENT OF ETHYL IODIDE IN ARTERIAL BLOOD MAY BE ESTIMATED FROM THAT OF THE ALVEOLAR AIR. Dogs were anesthetized by an intraperitoneal injection of 0.25 gram sodium barbital per kgm., more being given intravenously if necessary, and were placed on an electric warming pad. Carotid blood pressure was recorded and rectal temperature taken. Breathing through valves and a tracheal cannula, they inspired from the

spirometer a constant concentration of from 5 to 7.5 mgm. of ethyl iodide per liter of air, exhaling to the room. Satisfactory samples of alveolar air were secured from slowly breathing dogs by passing a small tube to the tracheal bifurcation and withdrawing about 5 cc. of air into a syringe at the end of each expiration. A mercury valve was found necessary to prevent the increase of pressure at the beginning of expiration from forcing dead space air into the syringe. When the dogs breathed rapidly we obtained alveolar air by a method somewhat similar to that employed by

TABLE 3

Comparison of the ethyl iodide concentration in arterial blood of dogs with that estimated from the concentration of alveolar air

DATE, 1927		DURATION OF INHALATION	ETHYL IODIDE CONCENTRATION		ERROR OF ESTIMATION	
			Estimated	Found by analysis		
		minutes	*mgm. per 5 cc.*	*mgm. per 5 cc.*	*mgm.*	*per cent*
February 16	A	24	0.67	0.63	+0.04	+6.3
	B	30	0.72	0.66	+0.06	+9.1
February 23	A	8	0.56	0.63	−0.07	−11.1
	B	16	0.74	0.75	−0.01	−1.3
March 3	I A	9	0.35	0.33	+0.02	+6.1
	I B	15	0.47	0.40	+0.07	+17.5
	II A	10	0.47	0.50	−0.03	−6.0
	II B	14	0.51	0.53	−0.02	−3.8
	III A	79	1.42	1.37	+0.05	+3.7
	III B	84	1.40	1.38	+0.02	−1.4

Mean difference = +0.013
Average deviation from mean difference = 0.036

In the experiment of February 16 alveolar air was collected from the end of each expiration: In the remainder by chest compression.

Inhalation of ethyl iodide was continued between the taking of samples A and B.

Krogh (1909) in rabbits. At the height of inspiration the inspiratory tube was clamped and the dog's chest compressed with both hands, driving air through the expiratory valve which then seated, preventing inspiration until 30 cc. of air were withdrawn from the tracheal tube. Respiration was interrupted for only a few seconds. The procedure caused a sharp fluctuation in blood pressure but the original level was regained within a few seconds. To secure sufficient air for analysis, this procedure was repeated three times in two minutes while the blood sample was slowly withdrawn from the femoral artery.

The distribution coefficient was determined upon the blood of each dog at the rectal temperature during the collection of samples. The concentration of ethyl iodide in arterial blood was estimated by multiplying that of alveolar air by the distribution coefficient, and is compared with the concentration found by analysis in table 3. The results demonstrate that the distribution of ethyl iodide between alveolar air and arterial blood has the same ratio as between air and blood in vitro.

To compare the ethyl iodide in alveolar air and arterial blood of human subjects inhaling the concentration used in blood flow determinations, a further series of experiments was performed. The mouthpiece shown in

TABLE 4

Comparison of the ethyl iodide concentration of human blood from the radial artery, or from a hand vein after immersion in water at 45°C. with that estimated from the concentration of alveolar air

DATE	SUBJECT	SOURCE OF BLOOD	FOUND BY ANALYSIS	ETHYL IODIDE IN ARTERIAL BLOOD			
				Estimated from individual distribution coefficient		Estimated from average normal distribution coefficient = 6.1	
				Error		Error	
			mgm. per 50 cc.	*mgm. per 50 cc.*	*per cent*	*mgm. per 50 cc.*	*per cent*
January 24	G.	A	0.66	0.72 (6.7)*	+9	0.66	0
January 26	W.	A	0.53	0.68 (5.8)	+28	0.73	+38
February 6	T.	A	0.59	0.61 (6.5)	+3	0.58	−2
February 8	C. S.	A	0.64	0.57 (5.6)	−11	0.63	−2
January 19	S.	V	0.40	0.38 (6.1)	−5	0.42	+5
January 31	H.	V	0.49	0.48 (5.9)	−2	0.51	+4

Omitting W, the average error using the individual distribution coefficient is ±0.04 mgm. or ±6.1 per cent; using the mean distribution coefficient for normals, the average error is ±0.01 mgm. or ±2.5 per cent.

* Figures in parenthesis indicate the coefficient determined on blood taken at the time of experiment (table 2).

figure 4 was similar in principle to that of Dill and others (1927), the expiratory valve being located inside the mouth. By the automatic method of Henderson and Haggard, modified as described below, the last few cubic centimeters of each expiration were drawn into the alveolar circuit for analysis. After the subject had inhaled ethyl iodide for about 12 minutes and 2.5 liters of air had passed through the sampling tube, the skin was anesthetized, the radial artery punctured, and 50 cc. of blood withdrawn into a syringe for determination of ethyl iodide content. To detect any changes in the respiration, the spirometer was read at half-minute intervals, and the rate observed. The concentration of ethyl iodide found by analysis was

compared with that estimated from the alveolar sample, using the distribution coefficient of blood taken at the same time.

In two subjects the same experiment was performed using blood drawn from a vein of the hand after prolonged immersion in water at 45°C. Goldschmidt and Light (1925) have shown that such blood closely resembles arterial blood in its gas content. In our experiments the oxygen saturation of this blood was as high as that of the samples taken from the radial artery of the other subjects.

The results of six consecutive experiments are recorded in table 4. In five of the determinations the results show that the arterial ethyl iodide concentration as estimated from the alveolar air and the individual distribution coefficient was correct within the analytical error involved. If the average distribution coefficient for normal human blood, 6.1, is used the agreement is even more striking. In the experiment on W. agreement was not obtained. This subject had great difficulty in adapting himself to breathing through the mouthpiece; he appeared cyanotic early in the experiment and was uncomfortable throughout. The respiratory record was irregular. Just before the sample was taken the subject had a period of coughing, his respiration increasing from six to eleven liters per minute. After the artery was entered the ventilation fell to four liters per minute and the blood drawn had a venous rather than arterial color. Therefore, we regard the low ethyl iodide content of this blood as due to hypoventilation during its withdrawal.

The objection of H. Barcroft (1927) to the automatic collection of alveolar air is not sustained under the conditions of our experiments.

We conclude that the ethyl iodide content of arterial blood of man may be estimated from a sample of alveolar air collected automatically under these conditions, using the distribution coefficient determined in vitro.

EXPERIMENTS ON DOGS INHALING ETHYL IODIDE, DEMONSTRATING THAT THE ETHYL IODIDE CONTENT OF MIXED VENOUS BLOOD REMAINS NEARLY CONSTANT DURING REBREATHING AND MAY BE ESTIMATED FROM THAT OF THE REBREATHED AIR. Dogs were prepared as described above. A flexible catheter was passed down the left jugular vein until its opening lay in the great veins opposite the auricular entrance, its position being verified by dissection at the end of the experiment. After breathing ethyl iodide for several minutes a peritoneal membrane bag containing 150 cc. of air was attached to the tracheal cannula; a sample of venous blood was taken immediately before making this attachment. During rebreathing additional samples of venous blood were taken until the blood pressure showed signs of collapse.

One experiment in which the samples were analyzed for oxygen, carbon dioxide and ethyl iodide is shown in figure 1. Other results are recorded

in table 5. They show that during rebreathing the ethyl iodide content of venous blood of dogs remains constant or falls very slowly.

With the same experimental arrangement dogs were made to rebreathe for from 20 to 50 seconds, immediately after the taking of a sample of mixed venous blood. A comparison of the ethyl iodide found by analysis in venous blood with that estimated from the rebreathed air and distribution coefficient is given in table 6. The experiments fall into two groups. In

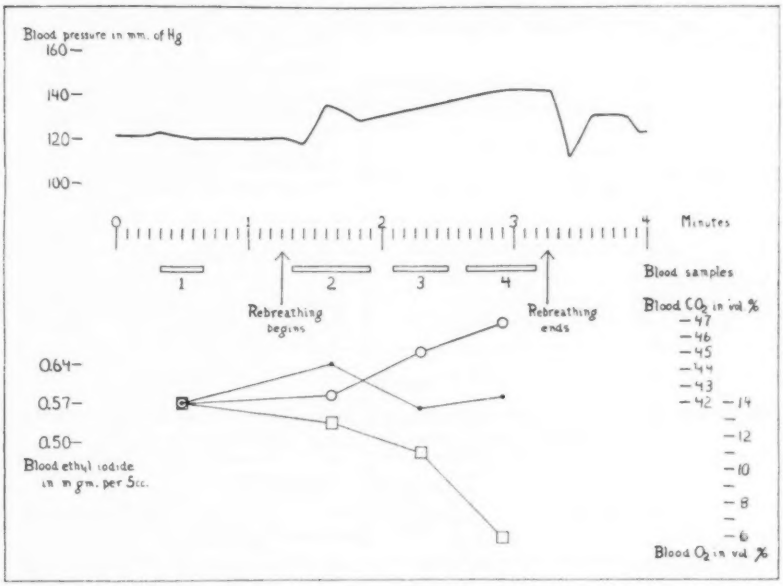

Fig. 1. The concentration of oxygen, carbon dioxide, and ethyl iodide in mixed venous blood of an anesthetized dog during rebreathing.

Dots = ethyl iodide; circles = carbon dioxide; squares = oxygen.

The scales are adjusted so that the values for the first samples are superimposed.

those of 11–26, 12–20 and 3–3, the coefficient of distribution was determined on the blood of the dog, at its temperature during the experiment. In the earlier experiments the average coefficient for dogs' blood at 37°C., 10.7 was employed.

Although there is marked divergence in one experiment, the agreement in eight of the twelve is within the analytical error involved and the averages agree well. We conclude that the experiments justify this method of estimating the ethyl iodide of mixed venous blood.

TABLE 5

Comparison of ethyl iodide concentration of mixed venous blood of dogs before and during rebreathing

DATE		TIME IN RELATION TO BEGINNING OF REBREATHING	ETHYL IODIDE
			mgm. per 5 cc.
December 15	A	Just before	0.79
		40–65 seconds after	0.75
		115–130 seconds after	0.67
	B	Just before	0.40
		30–45 seconds after	0.43
		75–91 seconds after	0.40
		105–120 seconds after	0.42
December 24		Just before	0.37
		70–110 seconds after	0.33
December 30	A	Just before	0.40
		25–35 seconds after	0.40
	B	Just before	0.37
		35–45 seconds after	0.32

TABLE 6

Comparison of the ethyl iodide concentration in mixed venous blood of dogs, with tha estimated from the concentration of rebreathed air

DATE, 1926–27		DURATION OF REBREATHING	ETHYL IODIDE CONCENTRATION		ERROR OF ESTIMATION	
			Estimated	Found by analysis		
		seconds	*mgm. per 5 cc.*	*mgm. per 5 cc.*	*mgm.*	*per cent*
November 3		45	0.20	0.20	0	0
November 9		30	0.43	0.44	−0.01	−2.3
November 16	A	30	0.19	0.22	−0.03	−13.6
	B	20	0.54	0.53	+0.01	+1.9
	C	20	0.52	0.62	−0.10	−16.1
	D	25	1.07	0.85	+0.22	+25.9
November 26		25	0.98	1.10	−0.12	−10.9
December 30	A	45	0.38	0.41	−0.03	−7.3
	B	50	0.37	0.37	0	0
March 3	A	30	0.35	0.34	+0.01	+2.9
	B	30	0.42	0.44	−0.02	−4.5
	C	30	1.16	1.02	+0.14	+13.7

Excluding 11–16, D, mean difference = −0.014 mgm.
Average deviation = ±0.044

EXPERIMENTS ON MEN PROVIDING EVIDENCE THAT THE ETHYL IODIDE CONTENT OF MIXED VENOUS BLOOD CAN BE ESTIMATED FROM THAT OF REBREATHED AIR. The comparison of the ethyl iodide content of mixed venous blood with that of rebreathed air could not be repeated in man. It seemed, however, that the indirect method of estimation shown to be correct for dogs, could be applied to man if it could be demonstrated that rebreathed air comes into equilibrium with venous blood and that the ethyl iodide concentration in the latter does not change significantly during the time necessary for equilibration. To test these points two samples of re-

TABLE 7

Ethyl iodide concentration in air rebreathed by man from a series of bags

DATE	SUBJECT	TIME AFTER BEGINNING OF REBREATHING			
		30 seconds	60–65 seconds	90–95 seconds	115–130 seconds
		mgm. per liter	*mgm. per liter*	*mgm. per liter*	*mgm. per liter*
February 17	S.	0.62		0.60	
February 22	C. S.	0.64		0.66	
	H.	0.48		0.47	
February 23	S.	0.62		0.51	
	G.	0.65		0.67	
February 24	W.	1.23		1.00	
	T.	0.81		0.84	
February 25	LaP.	0.90		0.76	
February 27	Hu.	0.80		0.76	
	Wo.	0.75	0.71		
	R.	0.64	0.60		
	S.	0.59	0.56		
February 28	Sc.	0.33		0.30	
February 29	W.	0.95	0.85*		0.68
	J.	0.63		0.61	0.57

Average decrease 30 to 90 seconds in 11 determinations = 5.6 per cent

* Sample taken 75 seconds after start of rebreathing.

breathed air were compared, the first taken after 30 seconds of rebreathing from one bag, and the second after an additional 60 seconds of rebreathing from another. It was arranged that the initial concentration in the rebreathing system should be above the final value in the first rebreathing and below it in the second. Failure to attain equilibrium or diminution in venous ethyl iodide would result in a lower concentration in the second sample. But if the final concentration in the two bags was the same it would prove both that equilibrium had been attained and that the venous ethyl iodide remained unchanged.

Fourteen experiments were performed on twelve subjects. After inhaling for 15 minutes the concentration of ethyl iodide used for blood flow determinations, the subject expired normally, inhaled from a bronze bag containing a liter of oxygen and rebreathed for 30 seconds, ending with a maximal expiration into the bag. He then inhaled from another bronze bag containing 1.5 liters of oxygen and about 0.7 mgm. of ethyl iodode, rebreathing from it for about a minute longer and ending with a maximal expiration into the bag. Samples of a liter of air were taken from each bag as soon as rebreathing ceased. While the first of the two bags contained no ethyl iodide, that in the lung air was sufficient to bring the initial concentration above that of equilibrium. The small quantity added to the

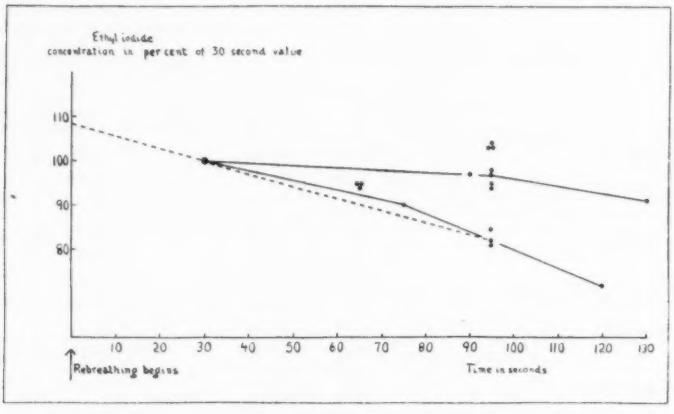

Fig. 2. Ethyl iodide concentration in rebreathed air of men during rebreathing, expressed as percentages of the value found at the end of 30 seconds' rebreathing.

second bag to prevent undue delay in equilibration, left its concentration below that found at the end of rebreathing.

The results are shown in table 7. In eleven of the experiments the concentration in the two bags agreed within the analytical error, the average concentration in the second bag being 98 per cent of that in the first. Since in these experiments equilibrium was attained, and the venous blood did not change between 30 and 90 seconds of rebreathing, it may be confidently assumed that the ethyl iodide in venous blood remained at the concentration it had before rebreathing began, as it does in dogs.

In four experiments the concentration in the second bag is clearly below that of the first, and in these the constancy of venous ethyl iodide content for the first 30 seconds of rebreathing cannot be so confidently assumed. The curve of venous ethyl iodide concentration during rebreathing may be

represented by a line joining the points obtained and evidence of the concentration before rebreathing may be obtained by extrapolation. Thus the straight line joining the points of one of the most divergent results crosses the ordinate at about 109 (fig. 2), suggesting that the sample obtained by rebreathing for thirty seconds is 9 per cent too small, which would make an error of about 4.5 per cent in determining the blood flow in this experiment. But the true curve of ethyl iodide concentration in venous blood during the rebreathing cannot be a straight line; the portion before one circulation time must be horizontal, then, as blood returns to the lungs in increasing amounts, the curve must bend downward. Therefore, the error must be much less than the straight line indicates. If no blood returns a second time within fifteen seconds, as is generally assumed, the error in estimating blood flow in this subject cannot be larger than 2.5 per cent and it is probably much smaller than this.

These findings and the experiments described in the succeeding paper lead us to consider the behavior of ethyl iodide in the body as resembling that of indifferent gases. During its inhalation its tension in venous blood represents an average of that of the tissues from which the blood returns, being lower than that of arterial blood because saturation has not been attained. During rebreathing the content of venous blood decreases slowly, the change being due to slow destruction, and probably to accumulation of ethyl iodide in lipoid tissues. Therefore, the rate contrasts markedly with the rapid and progressive change in O_2 and CO_2 resulting from metabolism.

From the foregoing experiments we regard it as proper to assume that the principles demonstrated in the dog experiments apply to man, viz., that the ethyl iodide content of mixed venous blood does not change materially during 30 seconds' rebreathing, and that this content may be calculated from rebreathed air and the distribution coefficient.

PERFUSION OF DOGS' LUNGS FOR THE PURPOSE OF COMPARING THE PERFUSION RATE WITH THAT ESTIMATED BY ETHYL IODIDE. Using these indirect methods of estimating the arterial and the venous content of ethyl iodide, the accuracy of which is supported by the experiments just described, we next attempted to estimate the flow through a dog's lungs perfused at a known rate. The experimental arrangement is shown in figure 3.

The dogs were anesthetized with sodium barbital. A tracheal cannula was inserted. Under artificial respiration from a Meyer pump the chest was opened in the midline, the pericardium split and a ligature placed around the pulmonary artery. A curved glass cannula was inserted into the right auricular appendage and tied in with a rubber band. Another cannula was placed in the left auricular appendage. When all was in readiness the curved cannula was pushed through the auricle and ventricle into

the pulmonary artery and tied in place. By starting the perfusion before this cannula was pushed into the pulmonary artery, interruption of flow through the lungs was avoided. The flow from the lungs was siphoned out through the left auricular appendage.

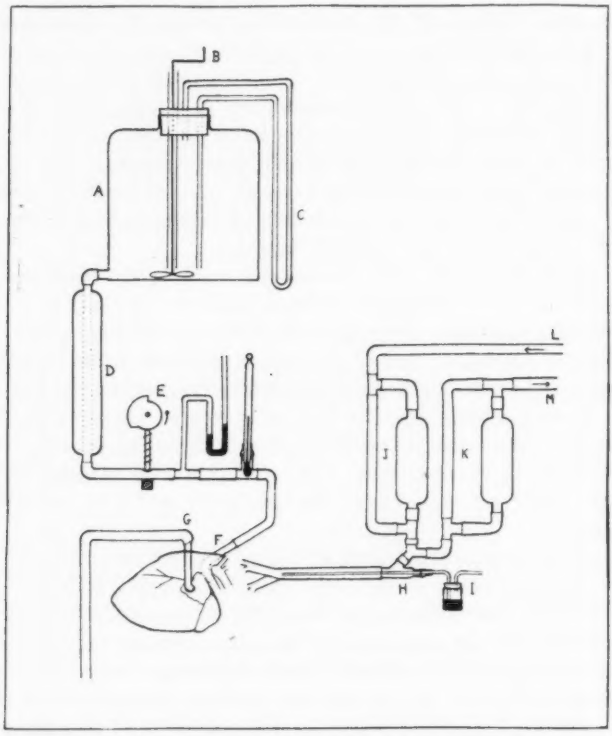

Fig. 3. Diagram of apparatus used in perfusion experiments. The heart and lungs remained in situ. *A*, perfusion bottle. Its water bath and heater are not shown. *B*, stirrer. *C*, gauge. *D*, tube with vacuum jacket. *E*, cam of interrupter. The motor is not shown. *F*, cannula through right auricular appendage, tied in pulmonary artery. *G*, outflow cannula and siphon. *H*, tracheal cannula. *I*, tracheal tube and mercury valve. *J*, inspiratory sampling tube and bypass. *K*, expiratory sampling tube and bypass. *L*, tubing from spirometer and Meyer pump. *M*, tubing to pump and room.

A solution containing 6 per cent gum acacia and 0.9 per cent sodium chloride was twice filtered through paper and used as perfusion fluid for the first experiment. In the second, third and fourth experiments defibrinated dog blood, filtered through paper, was added to the acacia solution, and in

the last experiments mixed defibrinated blood from several dogs was used alone. Agglutination of corpuscles did not occur. When the perfusion fluid contained blood a pulsating flow was secured by intermittent compression of the inflow tube, about sixty times a minute, by a motor-driven cam. The perfusion pressures varied from 26 to 40 mm. Hg in the various experiments. During the perfusion, air containing ethyl iodide and 5 per cent of carbon dioxide was pumped from the spirometer into the lungs, and the level of the fluid in the perfusion bottle and the position of the spirometer were read at frequent intervals. A sample of alveolar air was obtained through a tube extending to the tracheal bifurcation as described. After samples of alveolar, inspired, and expired air had been taken, the pump was disconnected and the lungs made to rebreathe by alternately drawing air into and

TABLE 8

Comparison between the rate of perfusion of dog's lungs as determined by ethyl iodide and by direct measurement

DATE, 1927	ETHYL IODIDE CONCENTRATION				RESPIRATION	DISTRIBUTION COEFFICIENT	PERFUSION RATE		
	Inspired	Expired	Alveolar	Rebreathed			Estimated by ethyl iodide	By direct measurement	
								Last minute	Entire experiment
	mgm. per 100 cc.	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*liters per minute*		*liters per minute*	*liters per minute*	*liters per minute*
April 11	1.59	1.44	1.17		4.4	1.5	0.38	0.36	0.38
April 19	1.85	1.73	1.56	0.34	5.0	2.1	0.23	0.22	0.18
May 5 A	1.19	1.17	0.95		4.8	3.3	0.03	0.38	0.45
May 5 B	1.32	1.20	1.11	0.29	5.0	3.3	0.22	0.28	0.29
May 12	1.91	1.69	1.13		3.5	9.2	0.64	0.69	0.57
May 13	1.81	1.57	1.03	0.87	3.5	8.6	0.61	0.60	0.63

expelling it from a 100 cc. syringe. The perfusions were of about four minutes duration.

Gross pathological examinations of the lungs after the experiments were always negative, as were microscopical examinations of sections from the last three. There was no evidence of pulmonary edema.

The perfusion experiments did not reproduce the repeated passing of the same blood through the lungs which is present in the normal circulation. In three of them, however (II, IV and VI), the perfusion fluid contained ethyl iodide as does the venous blood during a blood flow determination, having absorbed it in a previous perfusion.

The results are shown in table 8. In experiment III there was a large error, little ethyl iodide being absorbed from the respired air. This was probably due to improper adjustment of respiration, the lungs containing

too little air. This was corrected and experiment IV was performed on the same animal. In the 15-minute interval between the two perfusions the lungs were continuously ventilated, but were without circulation. In five of the six experiments the agreement between the estimated and observed flow is well within the analytical error.

The ethyl iodide distribution coefficient of the perfusion fluid varied from 1.5 for 5 per cent acacia in 0.9 per cent NaCl at 41°C. to 9.2 for defibrinated blood at 35°C. That it was possible to estimate blood flow over this range gives confidence in the principle involved.

AN IMPROVED METHOD FOR THE ESTIMATION OF BLOOD FLOW IN MAN BY ETHYL IODIDE. *The apparatus* described by Henderson and Haggard (1925) has been modified as follows:

1. To collect samples of rebreathed air a bag made of phosphobronze 0.005 inch thick is employed. Two sheets of this, each 12 × 24 inches, soldered together at the edges give a satisfactory capacity, holding two liters without undue pressure. Grease must be removed by repeated washings with ether. Even after careful preparation a loss of ethyl iodide from the bag of about 3 per cent in ten minutes was found.[2] Therefore samples are transferred to glass sampling tubes immediately.

2. The mouthpiece and valves are illustrated in figure 3. They are made from brass tubes of the following outside diameters in inches: no. 1, $1\frac{1}{4}$; no. 2, $1\frac{1}{8}$; no. 3, $\frac{3}{4}$; no. 4, $\frac{1}{4}$; no. 5, $\frac{15}{16}$; no. 6, $\frac{13}{16}$. The outer tube no. 1 and tube no. 2 are machined to fit accurately together; a set screw passing through a slot in the outer permits rotation through 180°. The L-shaped inner tube, no. 3, and the central tube, no. 4, for the collection of alveolar air are soldered to tube no. 2 and rotate with it. Turning the handle 180° closes *I* and *E*, the openings for inspired and expired air, and opens *R* for rebreathing. The expiratory valve (shown at the right, rotated through 90°) fits accurately into tube no. 5, which with its collar may be separated from tube no. 3 for cleaning and inspection. The inspiratory valve, which is not shown, is located beyond *I*. Because of the solubility of ethyl iodide in lipoids, grease must not be used for lubrication. Water or glycerin may be employed. The added dead space, i.e., the space between the teeth of the subject and the point from which the alveolar air is collected, measures 7 cc.

3. The alveolar circuit[3] has been modified by placing a Bohr meter beyond the sampling tube and by enlarging the Müller valve. The glass bulb

[2] The loss from celluloid bags is more rapid and from rubber or paper bags extremely rapid.

[3] A method for automatic sampling of alveolar air has been described by Wright and Kremer (1927), and a technique for securing "Haldane" samples during a determination of blood flow by Moore, Hamilton and Kinsman (1926). We know of no

is 2.5 inches in diameter and the end of the tube dipping below the surface of the water has been enlarged to a funnel, an inch in diameter. The desired rate of flow in the alveolar circuit is secured with a negative pressure of about 3 mm. of water in the spirometer and 6 mm. at the mouthpiece during inhalation.

4. To decrease the analytical error sampling tubes containing 500 cc. are employed. To shorten the time of exposure to water during collection, the outflow tube has been extended to the level of the floor. For the *I*[4] and *R* samples this tube dips below the surface of the water in the receiving vessel, preventing a negative pressure from diluting the sample with room air.

5. A fan has been placed in the mixing bottle for expired air.

6. Rubber parts of the apparatus have been reduced to a minimum, being used only to connect glass tubing and for the inspiratory valve.

PROCEDURE. The spirometer is filled with air containing approximately 1 cc. of ethyl iodide per 300 liters. After thorough mixing, enough is expelled to fill the tubing with the vapor. The subject's nostrils are closed with a noseclip. He then begins to breathe through the mouthpiece, the time being noted. During the experiment room temperature, time of taking samples, pulse and respiratory rates, and frequent readings of the spirometer are recorded. The rate of flow through the alveolar circuit is adjusted so that from 10 to 20 cc. of air are taken in at each breath. If the respiratory rate is rapid the lower limit should be approximated; if slow, the upper.[5] After two liters or more have passed through the meter, and after at least 12 minutes have elapsed, *I* and *E* samples are collected. As soon as 500 cc. more have passed through, the alveolar circuit is closed. Promptly thereafter at the end of a normal expiration, the valve is turned allowing the subject to breathe from the bronze bag containing 1 liter of air, the time being recorded. After 30 seconds of rebreathing the valve is turned at the end of an expiration and the *R* sample is taken from the bag. Two cubic centimeters of standard silver nitrate in nitric acid are then added to each sampling tube, and after 16 hours, titration is performed as described (Starr and Gamble, 1927).

CALCULATION AND CORRECTION. For a correct estimation of the blood flow through the lungs the simultaneous concentration of ethyl iodide in the

satisfactory fluid for the prolonged contact with the sample which these methods require. Mercury is inconveniently heavy, while water takes up significant amounts of ethyl iodide unless the displacement is rapid.

[4] The inspired, expired, alveolar, and rebreathed samples will be designated by the initial letters.

[5] In the arterial puncture experiments of table 4, 20 cc. per breath at 13 breaths per minute, and 10 cc. per breath at 17 breaths per minute gave correct alveolar samples.

inspired and expired air, and in arterial and venous blood should be known. Since the sample of rebreathed air cannot be taken until after that of alveolar air has been collected, and as the latter is not an instantaneous sample, a correction must be made for this delay. If we assume that the concentration of ethyl iodide in alveolar air rises at a constant rate (fig. 5) and that each portion taken mixes completely with the contents of the 500 cc.

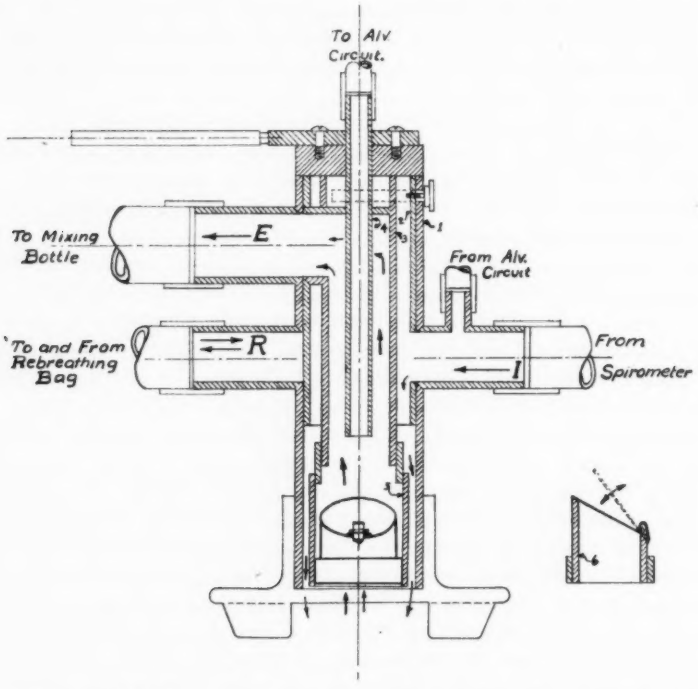

Fig. 4. Combined mouthpiece and valves. For description see text. Early in the investigation tube no. 1 was shorter than here shown, projecting only one inch beyond the side tubes; the expiratory valve then projected beyond it and was held within the subject's mouth.

sampling tube (a conclusion suggested by the fact that 2.2 liters of alveolar air were found to pass through before the CO_2 concentration became constant) it can be shown mathematically[6] that the concentration found in the tube when the alveolar circuit is closed is the same as that of the alveolar

[6] We are indebted to Dr. D. A. Flanders for assistance in the mathematics involved.

air at the time when the last 500 cc. began to flow through. This time, which represents the moment at which the blood flow is determined, will be designated as T. The I and E samples should be taken at this time, though because of the slow change in their concentration it need only be approximated. The number of minutes which elapse between T and the beginning of rebreathing should be used as the basis for correcting the concentration found in the R sample. In our experiments this has usually been between 2 and 3 minutes.

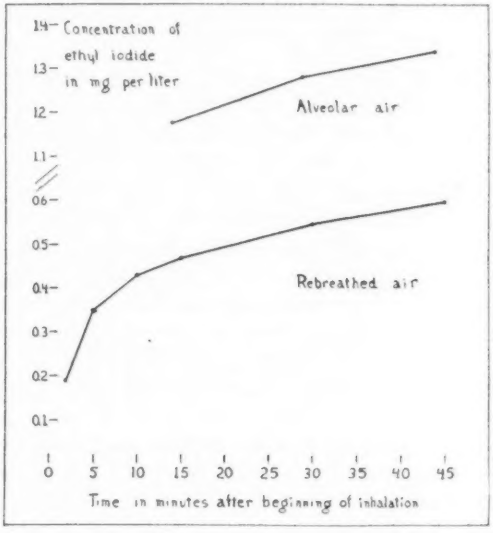

Fig. 5. The concentration of ethyl iodide in alveolar and rebreathed air of man during its continuous inhalation. The curve for alveolar air and the portion of that for rebreathed air from 15 to 45 minutes are derived from the average of estimations in the 5 triple blood flow determinations in table 9. The curve for rebreathed air from 2 to 15 minutes has been constructed from 4 other experiments, the results of each of which have been multiplied by the factor necessary to superimpose the values found at the end of 15 minutes' inhalation.

Four experiments (averaged in fig. 5) show that the rate of increase of ethyl iodide in venous blood between 10 and 15 minutes after beginning to inhale it averages about 1.5 per cent per minute for S, and 2 per cent per minute for G, supine and at rest. If inhalation is prolonged to 30 minutes, the rate of increase falls to 0.5 per cent per minute. These rates and the time between T and the beginning of rebreathing are used to calculate the corrections. In determinations subsequent to the first, the two measurements of the R value permit its estimation at T by interpolation.

Applying such a correction to the blood flow determinations of 3-13-28 on G, the R value found was reduced by 4 per cent in the first and 2.5 per cent in the second, while no correction was indicated in the third. Changes of −4, −2.5, and 0 per cent resulted in the calculated blood flow. Similar corrections for the experiments on S make less difference in blood flow val-

TABLE 9

Consecutive determinations of cardiac output of two normal men lying at rest

DATE	SUBJECT	ETHYL IODIDE CONCENTRATION (CORRECTED)				RESPIRATION	METABOLISM	PULSE RATE PER MINUTE	BLOOD FLOW THROUGH LUNGS
		Inspired	Expired	Alveolar	Rebreathed				
		mgm. per 500 cc.	*mgm. per 500 cc.*	*mgm. per 500 cc.*	*mgm. per 500 cc.*	*liters per minute*	*per cent of basal*		*liters per minute*
February 13	G.	2.74	1.27	0.63	0.23	6.8		68	3.9
March 13 (1)	G.	2.84	1.27	0.57	0.27	5.1	+7	64	4.1
March 13 (2)		2.96	1.33	0.72	0.36	5.65	+7	64	4.0
March 13 (3)		2.96	1.44	0.71	0.38	5.7	+8	60	4.1
March 17 (1)	G.	2.78	1.22	0.55	0.24	5.1	−3	64	4.0
March 17 (2)		2.87	1.25	0.63	0.29	5.3	+1	60	3.9
March 17 (3)		2.76	1.24	0.64	0.31	5.5	+3	58	3.9
March 8 (1)	S.	3.10	1.27	0.52	0.20	5.9	−11	75	5.3
March 8 (2)		3.20	1.22	0.65	0.27	5.85	−11	77	4.9
March 8 (3)		3.14	1.27	0.76	0.31	6.2	−3	77	4.1
March 15 (1)	S.	3.04	1.22	0.71	0.26	5.3	−8	68	3.4
March 15 (2)		3.05	1.31	0.60	0.27	5.5	−12	71	4.6
March 15 (3)		3.12	1.19	0.77	0.29	5.2	−17	72	3.3
April 24 (1)	S.	2.64	1.13	0.61	0.18	5.35	−4	72	3.0
April 24 (2)		2.63	1.16	0.63	0.19	5.35	−9	72	2.9
April 24 (3)		2.65	1.18	0.60	0.24	5.3	−14	73	3.5
May 22 (1)	G.	2.86	1.12	0.62	0.30	5.8	+19	68	4.9
May 22 (2)		2.86	1.18	0.65	0.34	6.15	+20	64	5.1

ues. In an individual determination the correction amounts to less than the probable error of the determination.

The concentrations found in the A and R samples, measured at room temperature are corrected to body temperature and saturation with water vapor.

Although from our present series of distribution coefficients of the blood

of 7 normal men (table 2) it appears reasonable to use the average value of 6.1 for any normal subject; this value is not applicable in anemic patients, nor in fever. It seems safer, therefore, to use the distribution coefficient determined upon blood taken at the time of the experiment, until more data have been accumulated.

The calculation follows, the I, E, A and R values being expressed as milligrams per liter, and the respiration and blood flow as liters per minute.

$$\frac{(I - E) \times \text{Respiration}}{(A - \text{corrected R}) \times \underset{\text{correction}}{\text{temperature}} \times \underset{\text{coefficient}}{\text{distribution}}} = \text{Blood Flow}$$

Results. Two individuals have been studied: G, aged 34, height 171 cm., weight 68 kgm.; and S, aged 33, height 183 cm., weight 79 kgm. Both are laboratory workers and exercise irregularly. Nine consecutive determinations on each are recorded in table 9. The last two determinations on subject G were made lying down one hour after lunch. In the others, the subjects had no breakfast and lay down on reaching the laboratory. Determinations on the same day were made without interrupting the breathing of ethyl iodide.

The distribution coefficients employed were the average of the four determinations on each subject recorded in table 2, G = 6.45, S = 6.25. Metabolism has been calculated according to the tables of DuBois from samples of expired air obtained from the mixing bottle. Oxygen consumed in fasting experiments varied from 220 to 263 cc. per minute for G, from 230 to 269 cc. per minute for S. In the experiment after lunch on G it amounted to 331 and 351 cc. per minute. Alveolar carbon dioxide tension in air taken from beyond the alveolar sampling tube varied from 39.8 to 42.0 mm. Hg for G, from 37.5 to 39.1 mm. Hg for S. The respiratory rates varied from 10 to 13 per minute for each subject.

The first series, on S, made after a preliminary rest period of forty minutes, showed a diminishing flow, so the rest period was lengthened to an hour in all subsequent experiments. The other determinations on S showed lower pulse rates, lower volume of respiration, and lower cardiac output, two determinations in each series agreeing, the third being significantly higher. In the case of G, whose metabolism determinations were more constant than those of S, all the blood flow determinations made while fasting agree within the error of the method. The consistency of these results on G is a source of confidence in the method.

Discussion. Various series of analyses of identical samples of air containing ethyl iodide indicate that our probable error of analysis of a single sample is less than 0.03 mgm. This quantity would introduce an error of about 6 to 7 per cent in the smallest of the quantities determined (A − R), and would therefore result in a probable error in an individual blood flow determination of about the same amount.

An advantage of the method just described lies in the absence of any procedure requiring voluntary coöperation. Compared with those using CO_2 or O_2, it has a greater proportionate difference between the arterial and venous concentrations, and a method of determining the content of venous blood which is much less dependent on the duration of rebreathing, and which has been shown to be correct in dogs. The fact that the determination is not limited to a single circulation time allows the subject to become accustomed to the conditions of the experiment before blood flow is determined.

In the method just described there is no procedure to disturb the circulation except rebreathing. The results of table 5 and figure 1 give evidence that in dogs the circulatory changes accompanying severe asphyxia cause no material change in the ethyl iodide content of venous blood. Therefore, if any alteration of the circulation occurs during rebreathing for thirty seconds, it would have no effect on the estimation of blood flow, the value obtained being the flow before rebreathing started. In the rest of the determination the oxygen consumption of our subjects shows no significant variation from that expected under basal conditions. Obviously no correction for change in oxygen consumption is necessary.

The method rests on the assumption that principles demonstrated in animal experiments are applicable to man, and on experiments on man which have increased our confidence in this assumption. We therefore believe that the method gives correct results on normal subjects, supine and at rest, and that its use can be easily extended to other conditions by further experiments of the type herein reported.

Although ethyl iodide has proved satisfactory for the purpose, it seems probable that other substances, after similar experimental tests, may also prove suitable for blood flow determinations with the apparatus and procedure described.

SUMMARY

1. The method previously described for the estimation of minute amounts of ethyl iodide in blood has been adapted for samples of 60 cc.

2. The distribution coefficient for ethyl iodide between air and blood at 37°C. is independent of changes of concentration from 1.2 to 48.0 mgm. per liter of air. The average value of seven normal human subjects is 6.1, varying from 5.6 to 6.5. Anemic blood has a lower coefficient, as has normal blood at febrile temperature.

3. The ethyl iodide content of arterial blood of anesthetized dogs can be estimated correctly from the content of a sample of alveolar air. The content of arterial blood of normal men supine and at rest can be similarly estimated from alveolar air collected automatically.

4. The concentration of ethyl iodide in the venous blood of dogs and in

the rebreathed air of man remains nearly constant during two minutes of rebreathing.

5. The ethyl iodide content of venous blood of dogs can be estimated correctly from the content of a sample of rebreathed air.

6. The rate of flow through dogs' lungs perfused at a known rate was estimated with satisfactory accuracy by means of ethyl iodide in five out of six experiments.

7. Based on the foregoing experiments a method for the determination of the rate of blood flow through the lungs of man, supine and at rest, is described, requiring no active coöperation. Consecutive determinations on two subjects are tabulated.

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THE BEHAVIOR OF ETHYL IODIDE IN THE BODY

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It was stated at the outset of the preceding paper that our experience with the Henderson and Haggard ethyl iodide method of measuring cardiac output in man was so much at variance with that of its authors that we felt obliged to abandon our original project of using it and chose to devote our efforts to the elaboration of another way of using ethyl iodide for the same purpose. In this paper we shall present the evidence upon which these decisions were based. The two questions which are of greatest importance are the fate of ethyl iodide in the body and the state of ethyl iodide in the blood, and under these two headings the greater part of our discussion will be presented.

I. THE FATE OF ETHYL IODIDE IN THE BODY. *Its presence in venous blood.* In their early experiments Henderson and Haggard (1925) found a very low concentration of ethyl iodide in the venous blood of dogs inhaling it, and recovered but little ethyl iodide from air rebreathed by men after ceasing to inhale it. A similar result in man was recently published by Mobitz and Hinsberg (1927) with the suggestion that the low concentration in exhaled air is derived from surfaces of the respiratory tract rather than from the venous blood. Henderson and Haggard concluded that hydrolysis, known to occur in watery solution, was so rapid in the body that it was practically completed in a single circulation time, and hence that the amount of ethyl iodide returning to the lungs was a negligible quantity.

The first communication expressing doubt concerning the completeness of this destruction was that of Moore, Hamilton and Kinsman (1926) who found that a significant amount of ethyl iodide was given off from the lungs after inhalation had ceased; they concluded that it must, therefore, be present in venous blood during the experiment. This was also found by ourselves (1927) and by Wright and Kremer (1927). We attributed the failure of Henderson and Haggard to find ethyl iodide in air rebreathed after a period of inhalation to its rapid absorption by the rubber of which the bags used to collect the samples were made. Employing a bronze bag we found large amounts of ethyl iodide in rebreathed air.

Henderson and Haggard (1927) now agree that ethyl iodide is present

in the venous blood of dogs for they have recovered it in significant quantity from blood taken from the right heart. Additional demonstrations of this are to be found in the 25 analyses of venous blood of dogs given in tables 5 and 6 of the preceding paper. That ethyl iodide is present in the venous blood of man as well as in that of dogs, during its inhalation, is shown by the following experiments.

The subjects inhaled ethyl iodide as for a determination of blood flow. Near the end of the determination 50 cc. of blood were withdrawn from the basilic vein, and the ethyl iodide content determined. In the first experiment (subject T) the inspired air contained 0.53 mgm. of ethyl iodide per cent (i.e., per 100 cc. of air) and the venous blood 0.37 mgm. per cent. In the second experiment (subject G) the inspired air contained 0.55 mgm. and the venous blood 0.54 mgm. per cent. Analysis of samples obtained three minutes before and four minutes after the venous punctures showed that the concentration of ethyl iodide in the expired air remained constant.

These results provide further evidence that the supposition of rapid and complete hydrolysis of ethyl iodide in the body is erroneous; that ethyl iodide is so consistently present in such significant amounts in the venous blood of dogs and of men that the factors of venous return of ethyl iodide to the pulmonary circulation cannot rightly be disregarded in estimations of the cardiac output by ethyl iodide according to the Fick principle.

The rate of destruction of ethyl iodide in the body. It is certain that ethyl iodide is hydrolyzed when added to shed blood. In determining the coefficient of distribution of ethyl iodide between air and blood in vitro, Henderson and Haggard encountered difficulties, the value of the coefficient apparently increasing with the duration of equilibration. As their analytical method determined not only ethyl iodide but also the alcohol produced by its hydrolysis, they concluded that there was a rapid destruction of the quantity dissolved in blood.

In our experiments, using a method of analysis which is not influenced by products of hydrolysis, destruction of ethyl iodide in blood in vitro at 37° was relatively slow, the loss amounting to only 26 per cent in 21 hours. This result has been confirmed (Kaup and Grosse, 1927). Hence we attributed our much higher values for distribution coefficient chiefly to increased saturation rather than to rapid destruction.

It may be conceded that the rate of destruction of ethyl iodide in vivo can hardly be less than that in shed blood. The observation of Mobitz and Hinsberg (1927) that iodine is eliminated in the urine of man during the 24 hours following inhalation of ethyl iodide may be interpreted as indicative of destruction in the body. But the actual rate of destruction in man has not been determined. The following experiments give a basis for estimating it.

After a test had shown that there was no loss of ethyl iodide in passage

through it, a large soda lime tube was inserted between the mouthpiece and the bronze rebreathing bag. The subject inhaled ethyl iodide for 15 minutes, as for a determination of blood flow, then rebreathed oxygen from the bag through the soda lime tube; further oxygen was added as needed; at intervals the subject rebreathed from a second bag of fresh oxygen while a sample was taken from the first. In each of two experiments on subject G, four samples were taken in succession, the first after 30 seconds rebreathing, the last after 7.5 minutes. These showed a decrease in ethyl iodide concentration which was approximately logarithmic at the rate of 11 to 13 per cent per minute, the value at 7.5 minutes being, in each experiment, 40 per cent of that at 30 seconds.

Since the rate of decrease in the ethyl iodide concentration in rebreathed air cannot be less rapid than the rate of its destruction in the body, we conclude that the rate of hydrolysis in these experiments was not greater than 13 per cent per minute; and since a part of the decrease in rebreathed air may have been due to a redistribution between lipoid-poor and lipoid-rich areas, it is not improbable that the rate of destruction was distinctly less than this.

The accumulation of ethyl iodide in the tissues. Henderson and Haggard believed that the relative constancy of ethyl iodide in the expired air observed by them during its inhalation indicated that its rate of destruction equalled its rate of absorption, and from this they concluded that none accumulated in the tissues. Similar observations have been reported by Mobitz and Grosse (1926). This conclusion was supported by an experiment by Henderson and Haggard (1925) in which they recovered no ethyl iodide from air which had been injected into the peritoneal cavity of dogs inhaling it; and Mobitz and Grosse were able to find only a trace of ethyl iodide in gas taken from a pneumothorax while the patient was inhaling it. Therefore, these authors agreed that ethyl iodide does not accumulate in the body.

In a later communication Henderson and Haggard (1927) concede that when conditions are established in which rate of absorption of ethyl iodide is very rapid (vigorous muscular exercise) accumulation may occur to such an extent as to invalidate the data for blood flow determination; but the inference to be drawn from their discussion is that under more usual circumstances such accumulation does not take place.

Accumulation of ethyl iodide in the body, if it occurs, must result in decrease in the rate of its absorption and increase in alveolar concentration; and these factors must necessarily yield decreasing values for the rate of blood flow as calculated by the method of Henderson and Haggard. Davies and Gilchrist (1927) actually found decreasing values for determinations made on the same day; they mention the possibility of such an accumulation, but prefer another explanation. Kaup and Grosse (1927) similarly

found decreasing values during the first 5 minutes of exercise and 20 minutes of rest, but concluded that a constant level, representing the correct blood flow value, was reached at the end of such a period. They attributed the change to a more rapid absorption of ethyl iodide in the earlier determinations, due to a gradual saturation of the blood, and denied its presence in the tissues. The results of others (Cullis, Rendel and Dahl, 1926; Rosen and White, 1926) show decreasing blood flow values in a number of experiments when the determinations are soon repeated. These results are consistent with the conception of a slow accumulation of ethyl iodide in the tissues when the subject is at rest.

Before describing the experiments which have forced us to conclude that ethyl iodide passes into tissues during its inhalation and accumulates there in increasing amounts, it is desirable that the results which we have obtained in repeating the experiment of Henderson and Haggard on air in the peritoneal cavity be stated.

1. A lean dog was chosen, anesthetized with sodium barbital and 200 cc. of air were injected intraperitoneally. After it had breathed 42 minutes from a spirometer containing 4.9 mgm. of ethyl iodide per cent, gas was withdrawn from the abdomen for estimation of its ethyl iodide content, and replaced by additional air. This procedure was repeated at the end of 113 and 153 minutes from the beginning of inhalation. The three samples were found to contain 1.37, 1.39 and 1.44 mgm. per cent of ethyl iodide.

2. Another dog was anesthetized with morphine and sodium barbital subcutaneously. Four hundred cubic centimeters of air were injected into the peritoneal cavity. Through a tracheal cannula he was then made to inhale ethyl iodide from the spirometer (about 4.9 mgm. per cent). Respiratory volume was 1 liter per minute and average blood pressure 85 mm. Hg. One hundred cubic centimeters of abdominal air were withdrawn after 8, 18 and 40 minutes of ethyl iodide inhalation. They contained 0.27, 0.22 and 0.58 mgm. ethyl iodide per cent. Mixed venous blood drawn from the right heart at the time of the last air sample, contained 8.5 mgm. per cent.

3. Thinking that the failure of Henderson and Haggard to find ethyl iodide in abdominal air might have been due to the state of shock said to be present, the animal used in the previous experiment was allowed to breathe fresh air for an hour, all remaining air having been withdrawn from the abdominal cavity. He was then bled until blood pressure fell to 30 mm. Hg. Respiration increased to an average of 3.5 liters per minute for the rest of the experiment. Four hundred cubic centimeters of air were again injected into the peritoneal cavity and inhalation of ethyl iodide again begun. One hundred cubic centimeters of abdominal air taken after 10 minutes' inhalation contained 0.25 mgm. of ethyl iodide

per cent; 200 cc. taken after 19 minutes' inhalation contained 0.40 mgm. per cent.

This consistent positive evidence of the presence of ethyl iodide in abdominal air of animals breathing it deprives, for us, the negative results which Henderson and Haggard obtained in similar experiments of the force which they seemed to possess. The reasons for this contradiction of evidence are still to be found. It should be noted that Henderson and Haggard (1925) originally used their finding that ethyl iodide was not present in abdominal air to support their theory of the rapid destruction of ethyl iodide in the body: more recently (1927) they used it as evidence that ethyl iodide of venous blood is in combination and hence does not exert significant partial pressure; for obvious reasons, we are unable to agree with either of these conclusions.

The positive evidence which convinces us that ethyl iodide accumulates in tissues is as follows:

1. After ethyl iodide inhalation is discontinued, venous concentration of ethyl iodide exceeds arterial concentration.

A dog, anesthetized with sodium barbital, was made to breathe for 40 minutes from a spirometer containing 4.4 mgm. per cent of ethyl iodide. Venous blood, obtained from a catheter to the entrance of the right heart, then contained 22.1 and arterial, 27.3 mgm. per cent. After breathing room air for 10 minutes simultaneous samples of venous and arterial blood contained 10.5 and 6.0 mgm. per cent.

In a similar experiment on another dog, after 40 minutes' inhalation of ethyl iodide, venous blood from the right heart contained 8.5 mgm. per cent. Fresh air was then inhaled for 10 minutes and venous and arterial samples taken simultaneously. Venous concentration was 3.8; arterial 0.3 mgm. per cent.

Since in both experiments, ten minutes after inhalation had ceased the venous blood contained much more ethyl iodide than the arterial, its amount being equal to 47 and 45 per cent of that present at the end of inhalation, we conclude that ethyl iodide must have passed into the blood from the tissues.

2. In our determinations of blood flow in man under conditions of basal metabolism the rebreathed and alveolar concentration (recorded in table 9 and figure 5 of the preceding paper) show increasing values as inhalation continues; the change in expired concentration must necessarily be less, it is within the analytical error in many experiments. As all controllable conditions remained constant during these experiments we believe that these increasing values indicate a slow accumulation of ethyl iodide in the tissues, similar to that demonstrated in dogs, under the conditions of a blood flow determination.

3. The recovery of ethyl iodide from venous blood of subjects inhaling

it for a determination of blood flow and having a constant concentration in the expired air (p. 475), together with the evidence, submitted in the following section, that the ethyl iodide of venous blood exerts a tension against its environment strengthens the belief that there is a tension of ethyl iodide in the tissues under these conditions.

It will be noted that the concentrations of ethyl iodide inhaled in the experiments upon dogs were higher than those used in blood flow determinations on man. The statements made in paragraphs 2 and 3 above, based on experiments in which men inhaled concentrations used for blood flow determinations, represent confirming evidence that ethyl iodide also accumulates in the tissues under these conditions.

THE STATE OF ETHYL IODIDE IN BLOOD. The discovery that ethyl iodide is present in venous blood of animals inhaling it led Henderson and Haggard to revise their original conception. In their recent paper (1927) they advance the view that ethyl iodide exists in circulating arterial blood partly free, partly combined, the latter possibly being within the corpuscles. That which is free is in simple solution in the water of the blood, is readily taken up by the blood and disappears completely during passage through the tissues; the combined ethyl iodide is slowly formed, slowly dissociated, exerts little partial pressure and is not rapidly destroyed. The concentration of free ethyl iodide in arterial blood depends upon its coefficient of distribution between air and water, which, according to the measurements of Henderson and Haggard, is 2; it may therefore be calculated by multiplying the alveolar concentration by this figure. Combined ethyl iodide is the chief or only form in which it exists in the venous blood; its concentration may be higher than that of the free, and hence it is responsible for the distribution coefficients which we, first, and Henderson and Haggard, later, obtained. Since it takes no part in the reactions upon which their method rests, it is, in this sense, negligible. With these assumptions, they regard their original calculations of blood flow as correct.

As supporting evidence for the validity of their assumptions a series of experiments on dogs anesthetized with morphine and cocaine is cited by them (Henderson and Haggard, 1927) which indicated that the difference between the concentrations of ethyl iodide in arterial and venous blood (i.e., the "free" ethyl iodide of arterial blood) is approximately double the concentration of ethyl iodide in alveolar air. With Brocklehurst (1927) they present a series of agreements between rates of blood flow in man as measured by the ethyl iodide method and simultaneously by a modification of Haldane's CO_2 method. From these, also, they deduced that the arterio-venous difference is approximately twice the concentration in alveolar air and are led to believe that this is a constant relationship.

We question most seriously the soundness of these assumptions. It can be conceded at once that ethyl iodide in blood may exist in different

concentrations in the plasma, in the corpuscles and in suspended fat droplets. Such is known to be true of chloroform, for example. But in the absence of direct proof there is certainly no more justification for assuming that this is due to a firmly fixed or slowly dissociated combination than to simple differences in the solubility of ethyl iodide in the various blood constituents. The latter assumption implies that while the concentration of ethyl iodide in blood plasma differs from that in blood corpuscles, the concentrations represent a solution equilibrium between two immiscible phases. As ethyl iodide is lost to the plasma either by destruction or by diffusion into tissues, it is supplied to it, pari passu, from the corpuscles, and this process by reason of the magnitude of the surfaces involved is rapid. The following facts not only give support to this simpler conception, but are definitely at variance with that of Henderson and Haggard.

1. When blood to which ethyl iodide has been added is distilled at reduced pressure all of the ethyl iodide comes off promptly. This observation has been made repeatedly in our estimations of known quantities added to blood, and the very fact that the coefficient of distribution between air and blood, determined in vitro, is the same whatever the absolute quantity of ethyl iodide taken (between 1.2 and 48 mgm. per liter of air) makes the probability of a combination, stable in the usual sense, seem very remote.

2. Complete equilibrium between blood and alveolar air with respect to ethyl iodide is reached during a single passage of blood through the lungs. In experiments in which surviving dogs' lungs were perfused with defibrinated blood (table 8 of the preceding paper) the rates of flow were correctly calculated by employing the distribution coefficients of 9.2 and 8.6 which had been determined in the usual way on samples of the perfusing blood. This shows clearly that the blood absorbed ethyl iodide to full equilibrium though it passed through the lungs but once. The process does not require two or three minutes for completion as believed by Henderson and Haggard (1927).

3. The ethyl iodide in venous blood exerts tension against gases in contact with it; nearly all the ethyl iodide in venous blood may be given off during a single passage through the lungs.

a. The experiments recorded in table 6 of the preceding paper show that when inhalation of ethyl iodide from the spirometer is discontinued and rebreathing begun, equilibrium is quickly attained between blood and rebreathed air at the same ratio as that which is constantly found between alveolar air and arterial blood, and also between air and blood in vitro.

b. Two experiments cited before provide additional evidence on this point.

An anesthetized dog inhaled ethyl iodide for 40 minutes; at that time blood from the right heart contained 8.5 mgm. per cent. He then inhaled

fresh air for 10 minutes and at the end of this time samples of arterial and of mixed venous blood were taken simultaneously. Arterial ethyl iodide concentration was 0.3; venous, 3.8 mgm. per cent. Ninety-two per cent of the venous ethyl iodide must have left the venous blood in a single passage through the lungs.

In another experiment on an anesthetized dog, alveolar air collected 8 minutes after inhalation of ethyl iodide had ceased contained 0.68 mgm. per cent. Arterial blood collected two minutes later contained 6.0 mgm. per cent. During the two minutes the arterial concentration must have decreased somewhat, hence the coefficient of distribution between alveolar air and arterial blood as estimated from these figures may be regarded as the same as the value 11.3 which was determined on this dog's blood in vitro.

These results show that there is no lack of free ethyl iodide in venous blood; they give no hint of a fixed form of ethyl iodide in blood; and they are strongly at variance with the conclusion of Henderson and Haggard (1927) that "dissociation after termination of inhalation seems to require considerable time, a half-hour or longer."

4. The difference between the concentrations of ethyl iodide in arterial and venous blood is not uniformly equal to twice the alveolar concentration of ethyl iodide. Ten experiments were made with dogs anesthetized with sodium barbital, in which a catheter had been passed down a jugular vein to the auricular entrance. Arterial and mixed venous blood were drawn simultaneously and the ethyl iodide contents determined. In seven of these the alveolar concentration was calculated from that of the arterial blood (a procedure justified by the results recorded in table 3 of the preceding paper). Expressing the results as ratios of the difference between concentrations in arterial and venous blood to concentration in alveolar air the following figures were obtained: 3.0, 3.3, 3.9, 2.1, 2.3, 2.1 and 0.4. In three other experiments in which the alveolar concentration was directly determined the ratios were 1.6, 1.9 and 3.1. The probable analytical error could account for the variation in the ratio of only about 0.6.

Similar variation is seen in the two experiments on man described on page 475. In the first the alveolar concentration of ethyl iodide was 0.17 mgm. and that of blood from the basilic vein 0.37 mgm.; in the second the alveolar was 0.13 mgm. and venous 0.54 mgm. per cent. Calculating the arterial concentrations from the alveolar air (a procedure justified by the results recorded in table 4 of the preceding paper) the ratio of arterio-venous difference to alveolar concentration in the first experiment was 3.9, in the second 2.3. While these figures obviously apply only to the area drained by the basilic vein, it is to be noted that the theory of Henderson and Haggard requires the arterio-venous difference to be the same for all veins.

While it is true that the average figure for the arterio-venous difference computed from the 10 experiments with dogs is approximately double (2.37) that of the alveolar concentration, it is certainly true that the use of the factor, Alveolar Concentration $\times$ 2, in calculating blood flow in five of the dogs and one of the men would lead to serious error. We conclude that the arterio-venous difference has no constant relationship to the concentration in alveolar air, but is influenced by a number of variable factors.

THE CONCORDANCE OF RESULTS OBTAINED BY ETHYL IODIDE AND OTHER BLOOD FLOW METHODS. The accuracy of the results of the method of Henderson and Haggard has been supported by the concordance of results obtained by it and other methods in animals and man. In eleven experiments on anesthetized dogs Henderson and Haggard (1925, 1927) obtained similar results in blood flow estimations by ethyl iodide and Fick methods. A single experiment on an unanesthetized calf reported by Mobitz and Grosse (1926) also yielded similar results by the ethyl iodide and the Fick methods.

In seven experiments on anesthetized dogs Henderson and Haggard (1927) found that the difference between arterial and venous ethyl iodide concentrations equaled twice the alveolar concentration. In the ten experiments cited above we found that the *average* arterio-venous difference equaled approximately twice the alveolar concentration, though the results in certain experiments varied greatly from the average. Therefore, we should expect the original ethyl iodide technique to estimate correctly the blood flow in many anesthetized dogs. But since the distribution coefficient for dog blood (averaging 10.7) is higher than that for man (averaging 6.1) we do not consider the results of experiments on animals to be applicable to man unless such differences be taken into account. The single experiment reported on the calf lacks force until it is confirmed and the similarity or difference between human and calf blood determined.

Brocklehurst, Haggard and Henderson (1927) obtained concordant measurements of the circulation in man by ethyl iodide and by a modification of the Haldane carbon dioxide method. Our limited knowledge of carbon dioxide methods does not permit any criticism based on personal experience. A survey of the literature discloses that the indirect methods for estimating gases of venous blood have not been compared with direct methods in either animals or man; and that the measurement of blood flow by carbon dioxide has never been successfully compared with direct measurement. There is always the possibility that in an occasional subject under certain conditions the difference between arterial and venous ethyl iodide might equal twice the alveolar concentration and in such a case the original technique would give a correct result.

ERRORS INHERENT IN THE ORIGINAL METHOD FOR THE ESTIMATION OF BLOOD FLOW BY MEANS OF ETHYL IODIDE. The facts and considerations which have been presented indicate that the method of Henderson and Haggard contains both systematic and non-systematic errors. Neglect of the ethyl iodide in venous blood introduces an error which is partially and to a varying degree compensated by the use of a distribution coefficient of 2 in calculating the content of arterial blood according to the original theory, or the arterio-venous difference according to the more recent one. The magnitude of this error varies greatly in different determinations under

TABLE 1

Comparison of blood flow estimations calculated from the same data by the method of Henderson and Haggard, and by the method described in the preceding paper

DATE	SUBJECT S		DATE	SUBJECT G	
	By method of preceding paper	By method of Hendersbn and Haggard		By method of preceding paper	By method of Henderson and Haggard
	liters per minute	*liters per minute*		*liters per minute*	*liters per minute*
March 8	5.3	9.9	February 13	3.9	7.4
	4.9	8.5			
	4.1	7.2	March 13	4.1	6.6
March 15	3.4	6.4		4.0	6.0
	4.6	7.5		4.1	5.7
	3.3	6.0	March 17	4.0	6.7
April 24	3.0	6.0		3.9	6.4
	2.9	5.9		3.9	6.1
	3.5	6.0	May 22	4.9	7.6
				5.1	7.5

These experiments are tabulated in detail in table 9 of the preceding paper.

Determinations bearing the same date were made during the continuous inhalation of ethyl iodide.

apparently similar conditions; it seems to depend in part on the subject. In eleven resting subjects (table 7 of the preceding paper, p. 461) after 15 minutes' inhalation of the concentration of ethyl iodide employed for the determination of blood flow (about 5.8 mgm. per liter), the amount found in rebreathed air varied from 0.33 to 1.23 mgm. per liter.

The period of inhalation of ethyl iodide has varied in different investigations and its limits have not been specified. The error due to neglect of venous ethyl iodide increases with the duration of inhalation, as is shown by the experiments which demonstrate progressive accumulation in the tissues (pp. 477–9) and in our experiments recorded in table 1. Successive

determinations of blood flow during continuous inhalation of ethyl iodide when calculated by the method of Henderson and Haggard from the data of table 9 of the preceding paper show that the blood flow apparently decreased progressively. When the calculations are made according to the method described in the preceding paper, no such progressive change, dependent upon duration of inhalation, is apparent. They have a lower average value.

Variation in the distribution coefficient, which we have found to be lower for anemic blood (table 2 of the preceding paper), introduces an additional error which may account for the failure of Mobitz (1926) and of Kininmonth (1928) to find increased blood flow in anemia.

SUMMARY

In previous work (Starr and Gamble, 1927) we found that the coefficient of distribution of ethyl iodide between air and blood is far higher than had been reported, and obtained indirect evidence of the presence of ethyl iodide in the venous blood of men inhaling it. We therefore concluded that the method of Henderson and Haggard (1925) for the determination of cardiac output in man was based on an erroneous conception and gave unreliable results.

In the meantime, Henderson and Haggard (1927), having accepted and confirmed this evidence, have reconstructed the theoretical basis of their method, introducing a conception of free and combined ethyl iodide in blood which, they believe, justifies them in disregarding the absolute value of ethyl iodide concentration in venous blood. Though their theoretical conceptions have changed, their confidence in the correctness of blood flow estimations made by the original technique has not diminished.

The evidence presented in this paper has forced us to conclude that this newer conception is also faulty. Ethyl iodide is rapidly and completely taken up and given off by blood passing through the lungs. Ethyl iodide is constantly present in the venous blood of men or dogs inhaling it and exerts pressure against gases in contact with it. The destruction of ethyl iodide in the body is slow and does not equal its rate of absorption under the conditions prescribed for the determination of blood flow. The difference between arterial and venous ethyl iodide concentrations bears no constant relationship to the alveolar concentration in dogs and men.

These results lead to the belief that ethyl iodide behaves in large part like an indifferent gas and distributes itself in blood and tissues according to its solubility in their various components. But because part of the ethyl iodide is destroyed in blood and tissues saturation of the body at the concentration inspired is delayed and the difference between arterial and venous concentrations remains large even after prolonged inhalation.

Therefore, still believing that the ethyl iodide in venous blood is a source of error in the Henderson and Haggard method, we have devised a technique for estimating it and believe that by its use the cardiac output of resting men can be determined by ethyl iodide according to the Fick principle.

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STUDIES ON THE PHYSIOLOGY OF THE LIVER

XVI. The Respiratory Quotient and Basal Metabolic Rate Following Removal of the Liver and Injection of Glucose

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When we discovered 1, that total removal of the liver in an otherwise intact animal was followed by a decrease in the blood sugar; 2, that the decrease in the blood sugar was accompanied by a group of characteristic symptoms (12), and 3, that glucose would relieve the symptoms and temporarily restore the animal to normal (13), three questions, corollaries to these observations, presented themselves; 1, the fate of the sugar which disappeared in the dehepatized animal; 2, the cause of the symptoms associated with hypoglycemia, and 3, the fate of the injected glucose and the cause for its beneficial but temporary action. It appeared that certain data that might possibly assist in answering these questions could be obtained by a study of the production of heat and respiratory quotient in the dehepatized animal and also the effect on these two factors of the injection of glucose into the dehepatized dog as compared with the dog possessing a liver. The first experiments on the problem were carried out in 1921, and a few additional experiments have been performed each successive year. The results of the first series of experiments were reported (11), but their publication has been delayed. The experiments were most difficult and it was difficult to secure trustworthy data. Gradually with the elimination of various sources of error through the development of better technic for training the animal, making the metabolic tests and removing the liver, the results of the experiments have become more uniformly satisfactory. We are now presenting our reports, not because the questions originally leading to it can be completely answered, but because sufficient trustworthy data have been secured to establish certain facts concerning the respiratory metabolism of the dehepatized animal.

Review of previous work. Several investigators have dealt with the effect of removal of the liver on the respiratory quotient. Practically all of these investigations were carried out for the purpose of obtaining data either for or against one of the important theories of the production of

diabetes mellitus, the theory of overproduction or the theory that glucose fails to burn. Some of the more recent investigations have as their principal objective a study of the effect of insulin on carbohydrate metabolism. The physiologic significance of the liver has usually not been the prime consideration in studies dealing with the respiratory quotient in the dehepatized animal.

Bohr and Henriques studied respiratory metabolism after ligation of the thoracic aorta, and in some instances also the inferior vena cava, in dogs and rabbits. The animals were curarized and respiratory ventilation maintained artificially; they lived only a short time after ligation of the vessels, and in some of the experiments an increase in respiratory quotient was noted. Scaffidi studied the gaseous metabolism of geese in which the portal vessels were ligated, but the liver was left in the body with intact arterial circulation. The fowls were observed for several days after operation. In some of the experiments an increase in the respiratory quotient was noted for a few days after operation. Scaffidi explained these results as owing to increased consumption of glucose because of failure to store glycogen.

Porges investigated the problem in rabbits. After a fast of from twenty-four to forty-eight hours the animal was given urethane, and when the anesthetization was complete, the abdominal aorta, vena cava, and the hepatic and portal veins were ligated. After a short wait to exclude the immediate effect of the operation, the respiratory metabolism was studied. Only a few of the experiments were successful. In these a considerable increase in the respiratory quotient was obtained after the circulation to the body below the diaphragm had been stopped. Porges interpreted the results as showing that after the liver is removed, only sugar is burned by the organs. Porges and Salomon performed some experiments on dogs in which the pancreas had been removed one or two days preceding ligation of blood vessels below the diaphragm. These animals also showed an increase in the respiratory quotient, and the investigators accepted this as proving that depancreatized dogs burn sugar.

Fischler and Grafe studied the gaseous metabolism of dogs before and after performing an Eck fistula. They observed a distinct elevation of the respiratory quotient from normal only in the stage of intoxication. Verzár cut off the portal circulation from the liver of dogs (Queirolo operation), and found that the respiratory quotient increased following the intravenous injection of glucose or starch. He concluded that carbohydrates can be burned without the liver, and that they do not need to be changed into glycogen.

Rolly repeated the experiments of Porges and obtained evidence in some of his experiments of a rise of respiratory quotient. He did not, however find the increase to be consistent, and attributed it to changes in reaction

of blood with loss of carbon dioxide. Böhm obtained only a slight rise in respiratory quotient after exclusion of the abdominal organs of depancreatized dogs.

Fischler and Grafe studied the respiratory exchange following ligation of the hepatic artery in dogs in which Eck fistula had been performed a

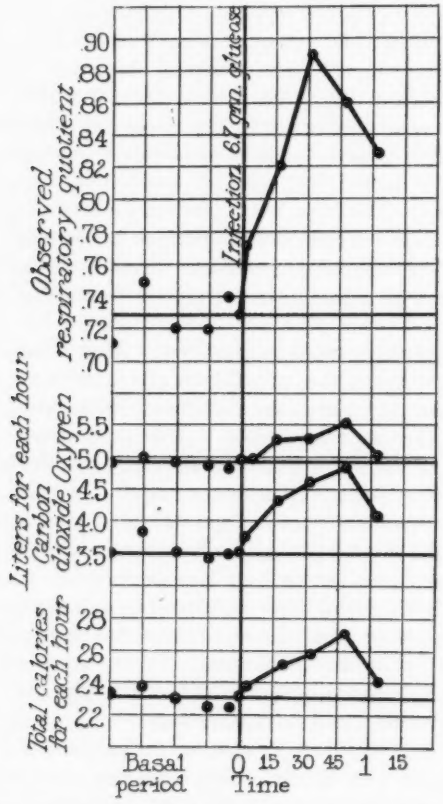

Fig. 1. The response of a dog before hepatectomy to 6.7 grams of glucose.

few weeks previously. In most of their experiments the respiratory quotient increased. This increase was particularly noticeable in the experiments in which the animal lived only a short time. In the experiments in which the animals lived for many hours after ligation of the hepatic artery, the increase in the quotient was usually a terminal event. Production of heat decreased after operation, although it also increased toward the end

of the experiment in the animals that lived longest. Fischler and Grafe were inclined to interpret their results as did Rolly, and not as supporting the conclusions of Porges.

Murlin, Edelmann and Kramer determined the carbon dioxide and oxygen content of the blood and the respiratory quotient after clamping the abdominal aorta and inferior vena cava below the diaphragm in normal dogs and after pancreatectomy. Their experiments were performed on normal dogs anesthetized with chloretone. In most of their experiments a marked decrease was noted in the carbon dioxide of the arterial blood, and in some instances a decrease in the oxygen content of the blood after occlu-

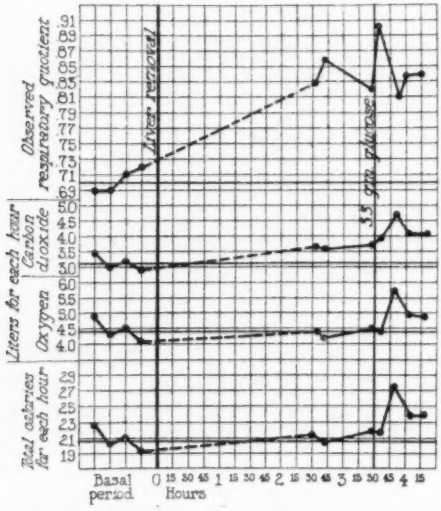

Fig. 2. The level of production of heat following removal of the liver is practically the same as the preoperative basal value. The injection of 3.3 grams of glucose gives a slightly greater response than 6.7 grams in the same dog before hepatectomy (fig. 1).

sion of the blood to and from the body below the diaphragm was also noted. In certain experiments the respiratory quotient increased; in others it decreased, and in the remaining experiments it did not change. The changes in the gaseous constituents of the blood, with particular reference to the loss of carbon dioxide, were consistent with the view that the changes in the respiratory quotient after clamping of the aorta and vena cava were due to the loss of carbon dioxide. These authors reviewed critically all previous work on the problem and concluded that obstruction of the blood to and from the abdominal organs does not alter the metabolism, and that all the results can be explained on a mechanical basis.

Grafe and Denecke studied the effect of extirpation of the liver on the temperature and respiratory exchange. They used geese and dogs. In the latter, they removed the liver by performing an Eck fistula, and a considerable time later removed the liver lobe by lobe. They found that the temperature and systemic processes were decreased following removal of the liver.

Burn and Dale determined the respiratory quotient of the decapitated and eviscerated cat in an investigation which was primarily for the purpose of studying the action of insulin. They found the respiratory quotient of an animal thus prepared, in which glucose was injected at a constant rate to maintain the blood sugar level, to be unity. Similar results with the

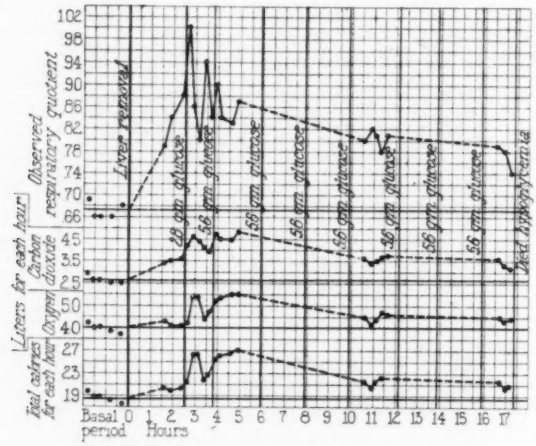

Fig. 3. Metabolism practically unchanged seventeen hours after hepatectomy. Death from hypoglycemia.

use of the same type of animal preparation were obtained by Best, Hoet and Marks.

Markowitz, employing the three-stage method of removing the liver, found that the administration of glucose to a dehepatized dog caused a prompt increase in the respiratory quotient to unity accompanied by an increase in the production of heat. When the animal was depancreatized forty hours before hepatectomy, the glucose did not have any effect. Nor was there a significant increase in the respiratory quotient following the administration of glucose several hours after total evisceration.

Kilborn studied the respiratory quotient in eviscerated, decapitated cats in which respiration was maintained artificially. He found it to be unity; this value was not due to combustion of carbohydrate by the muscle,

but to loss of carbon dioxide dependent on the fact that the lungs were being over-ventilated and that lactic acid accumulated.

It is evident from this review of previous work that there were many serious sources for error in most of the experiments. In many of the experiments the gaseous metabolism was that of a head-thorax preparation. All experiments were complicated with a narcotic or anesthetic some time during the experiment, and in few experiments was the operative procedure and the length of life of the animal such that the anesthetic could have been withdrawn for a sufficient period to allow equilibrium in a basal condition to become reëstablished. In most experiments the operative procedures were extensive, prolonged and either accompanied by loss of blood or large vascular areas. However, the general results of most of the experiments are in agreement in one particular, namely, the respiratory quotient increased after the loss of the liver.

METHOD OF EXPERIMENTATION. The method of determining gaseous metabolism in dogs was the method described in detail by Kitchen. It is an adaptation for use on dogs of the same technic that has been developed and fully described by Boothby and Sandiford for man. In experiments of the kind to be described in which it is essential to determine the respiratory quotient and production of heat over many successive short periods, and after various procedures, only the indirect method of calorimetry can be employed. The important considerations in adapting the method to the dog were: 1, careful selection of the animal; 2, accurate control of the diet; 3, careful training of the dog; 4, development of a special mask; 5 control of the environmental factors in the laboratory which might disturb the animal while under observation, and 6, carrying out of the metabolism technic described by Boothby and Sandiford.

Care was exercised in selecting animals that were quiet and seemed to be capable of being trained. They were placed on an approximately constant diet which would just maintain their body weight. They were always fed at approximately the same time each day which permitted a fast of approximately eighteen hours before beginning the succeeding tests. The training of the animal was carried out in successive steps. In the first period the animal was placed on the table for an hour or so, with feet held in place by a restraining strap. In the next period, the mask was placed on the head without being adjusted. Finally, the animal was allowed to lie quietly for hours at a time with the mask adjusted for observations. If at any stage of the training, the animal was found unfit for this special work, it was discarded. The mask used was a hollow cylindrical cone of copper sheeting, open at the base and having inlet and outlet tubes at the apex. The animal's head was placed in the mask and the opening around the neck made airtight with a carefully placed rubber-dam. It was found that even the best trained animals, although lying quietly, would respond

to unusual sounds, evidenced by change in pulse and respiratory rate. In order to avoid this source of error, extraneous noises were eliminated as completely as possible. The exterior temperature around the animal was maintained fairly constant by means of a box with a glass top which could be raised or lowered over the animal. Artificial heat was supplied to the box by a single electric globe. By proper adjustment, the environmental temperature was kept fairly constant. The routine for collecting the air, its analysis, and so forth, did not differ from the same kind of procedures as carried out on the human being. The time for collecting the air was usually ten or fifteen minutes. During this time the pulse, respiration, and environmental and skin temperatures were carefully observed. In most of the experiments a graphic record was made of the respiratory movements; the pneumograph used for this purpose was so adjusted that it likewise recorded even the smallest extraneous movements whenever these occurred, and in addition the animal was closely observed by an assistant. If movements occurred, the test was designated as unsatisfactory and not considered of value in drawing conclusions.

The routine procedure in the hepatectomy experiments was as follows: The two preliminary operations for removal of the liver according to our method of hepatectomy were performed. It should be noted that in the preliminary operations for removal of the liver, the portal blood is shunted away from the liver. Animals thus operated on with the exception of the observation mentioned later, responded as normal animals in regard to the metabolism tests. Therefore, the animals, before removal of the liver, will be spoken of as normal in comparison with the dehepatized animal. After the animal had been prepared for removal of the liver it was carefully trained for metabolism work. Several preliminary tests were carried out and when the successive tests checked closely and were basal values for that particular animal, the final experiments were performed. In most of the animals the effect of the injection of definite amounts of glucose was obtained preceding the final experiment. On the day of the final experiment, two or more tests were made; the animal was then etherized and the liver removed quickly. Tests were made at varying periods after hepatectomy, when the animal was hypoglycemic, and after the injection of varying amounts of glucose. Following the injection of glucose, tests were made at various short intervals. In some experiments tests were made almost continuously for several hours after the liver had been removed; in a few experiments tests were made as long as twenty-four hours afterward. Blood sugar determinations were made at varying intervals when the procedure of taking blood would not in any way disturb the animal for the metabolism tests. In some of the experiments the carbon dioxide combining power of the blood was determined before hepatectomy and at varying intervals afterward.

RESULTS. The observations were carried out in a sufficient number of satisfactory experiments, thirty in all, to allow certain general conclusions. Although all the experiments were satisfactory in some respects, not all were satisfactory throughout. In a few of the earlier experiments, the pre-operative period of observation was so short that the control data were deficient. In most of the experiments, however, the control observations made before removal of the liver were entirely adequate. In a few experiments tests were carried out two to three times a week for more than a year before the final experiment. As may readily be surmised, the highly technical character of the investigation made it exceedingly difficult to carry out the details. In order to obtain the best results in metabolism work on animals, it is exceedingly important to select the proper type of animal, because in order to obtain consistent results, the dog must be well trained. Since it was necessary to perform two preliminary operations before beginning observations, the range of choice was greatly decreased. This resulted in the use of animals which would not have otherwise been used for metabolism work. Some of the animals that had been satisfactory in the pre-operative observation became restless following anesthesia and removal of the liver. There was always the race between quieting the animal sufficiently for the metabolism tests and the development of hypoglycemia.

In most of the animals a sufficient number of satisfactory tests were made before the liver was removed. Since in all the animals prepared for removal of the liver the portal blood was shunted away from the liver, these data apply specifically to the animal on whom Eck fistula had been performed. In a few of these animals the basal respiratory quotients increased gradually. At first, we attributed this to the progressive atrophy of the liver which characterizes such animals. However, while it is possible that a hepatic factor was present, the cause for the high quotient was probably due to slow absorption from the gastro-intestinal tract because the quotient decreased when the period of fasting was increased. In general the results of the test checked fairly closely each day for any particular animal and were comparable to the tests on normal dogs.

The intravenous injection of glucose before removal of the liver produced surprisingly slight response. The injection of 0.25 gram of glucose for each kilogram of body weight caused little or no change in the total heat produced and the respiratory quotient was only slightly increased. When 0.5 gram of glucose for each kilogram was injected, the total heat produced was sometimes increased slightly and there was always a small but definite increase in the respiratory quotient. The administration of 1 gram or more of glucose for each kilogram of body weight always produced an increase in both the total heat and the respiratory quotient. These results are in every respect comparable to the results obtained in normal animals not operated on.

The respiratory quotient increased following removal of the liver in all experiments except in a few in which the quotient was not at the fasting value before operation. The quotient usually increased from the fasting level of 0.70 to 0.75 to 0.85 to 0.95. It rarely reached unity. There was always a further increase in the dehepatized animal following the injection of 0.25 gram or more of glucose for each kilogram of body weight. Such an increase was always apparent in the first test beginning within five minutes after the injection of the glucose. The response with regard to the respiratory quotient was greater following a given amount of glucose in the dehepatized animal when compared to the control injection preceding removal of the liver.

The total amount of heat produced did not change following removal of the liver in experiments in which extraneous factors were eliminated. In some experiments it varied, depending on concomitant factors other than the loss of hepatic tissue. If the animal was restless after operation the production of heat was increased. If the animal was hypoglycemic, with accompanying muscular twitching, an increase was noted. There was always an immediate increase in the production of heat following the injection of glucose. Here also the same amount of glucose would usually produce a greater increase in the total amount of heat produced after hepatectomy than before, as was noted in regard to the respiratory quotient.

The data on the carbon dioxide combining power of the blood and on the lactic acid in the blood after removal of the liver will be presented in full later. As regards these experiments, it must suffice to state that while the carbon dioxide combining power of the blood decreased following removal of the liver, this decrease was apparently associated with the anesthesia and not the loss of hepatic tissue, because it usually recovered its pre-operative level before the end of the experiment. In connection with the subject of this report it should be noted that the lactic acid content of the blood of the dehepatized animal varies considerably, and may or may not be greater than that of the normal animal. In this series of experiments it did not appear that the rise in respiratory quotient was due to any change in reaction of the blood and loss of carbon dioxide.

Comment. In attempting to evaluate the result of these experiments it should be recalled that the utmost care was employed to make the data obtained trustworthy. The animals were well trained for metabolism work and satisfactory pre-operative tests were obtained. In many experiments the pre-operative tests were made two or three times a week for several months before the liver was totally removed. The periods of anesthesia and operation were relatively short. Control experiments, sometimes performed on animals in which the liver was removed subsequently, demonstrated that basal conditions as regards gaseous metabolism would be established within the period subsequent to operation in which observa-

tions were made on the dehepatized animal. The dehepatized animals appeared normal at the time the tests were made. In some experiments the gaseous metabolism was followed for more than twenty-four hours after removal of the liver. All these considerations make it seem that the results obtained are thoroughly trustworthy and justify the statements based on them.

In general three facts stand out as the result of this study of the gaseous metabolism of the dehepatized animals: 1, the total production of heat is not directly affected by the loss of the hepatic tissue; 2, the respiratory quotient increases following the loss of the liver, and 3, the effect of the intravenous administration of glucose is greater in the dehepatized animal than in the normal animal, both as regards increase in respiratory quotient and in the total amount of extra heat produced.

The total heat produced by the dehepatized animal depended on its being quiet or restlesss, normal or hypoglycemic, and so forth. There was no evidence that the loss of the liver itself caused an increase in the production of heat and the limits of error in determination preclude the detection of a change in the quantity of heat produced that would be the result of the decrease in the mass of the active tissue removed. The metabolism as regards the production of heat appeared to be the same in the dehepatized animal as in the normal animals; the available fuel, therefore, was burned at the same rate even though the supply was soon to be exhausted. Evidently the liver does not have any direct control over the basal production of heat.

When due allowances have been made for the many possible complicating factors, the results of the experiments, the conclusion is evident that the total loss of the liver increases the respiratory quotient, and that this change is probably of metabolic significance. The conclusion is based on the fact that we have not been able to obtain sufficient data to implicate the possible complicating factors such as the anesthetic at operation, loss of carbon dioxide because of the accumulation of lactic acid or a change in the reaction of the blood due to other causes, although it must be admitted that there may be some unknown factor. The metabolic cause for this increase in quotient, if it is metabolic, is not clear. It might indicate that a greater proportion of carbohydrate was being utilized, although other explanations are possible.

It is also probable that the same amount of glucose will produce a greater increase in respiratory quotient and production of heat, that is, a greater specific dynamic action in the dehepatized animal than in the normal one. The simplest explanation for this fact would be that the liver of the normal animal takes up much of the injected glucose and thus prevents its immediate utilization. Here again, however, other explanations are possible.

The differences in the results obtained in our experiments and those of

some of the other investigators can probably be explained in the difference in the method of investigation. Most of the experiments were complicated by an anesthetic at the time the tests were being made or were performed on decerebrate or spinal animals. The liver was removed by evisceration, or a head-thorax preparation was used. In some of the investigations artificial respiration was employed. All these methods of procedure are complicating factors, making it difficult to obtain trustworthy data free from error, and the interpretation of the results is fraught with pitfalls.

SUMMARY

A series of observations on gaseous metabolism was made on trained animals before and after removal of the liver and before and after the injection of glucose. It was found that: 1, the total amount of heat produced was not directly affected by the total loss of hepatic tissue; 2, the respiratory quotient increased immediately after removal of the liver, and 3, glucose had a greater specific dynamic action in the dehepatized animal than in the normal one.

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STUDIES ON THE PHYSIOLOGY OF THE LIVER

XVII. The Effect of Removal of the Liver on the Specific Dynamic Action of Amino Acids Administered Intravenously

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References found in various ancient and medieval writings seem to indicate the recognition in a vague manner that food possesses a unique stimulating influence on the living organism and causes an increase in the level of the production of heat. Clear-cut quantitative studies were not made of this phenomenon, however, until toward the end of the eighteenth century when Lavoisier and Sequin demonstrated (in man) that the rate of oxygen consumption is greatly elevated above the resting level following the ingestion of food. The death of Lavoisier prevented further investigation of this phenomenon and further work of importance was not done until the nineteenth century, when the rise of the German school, headed by Rubner and his pupils, again stimulated interest in the subject. Rubner first applied the term, specific dynamic action, to this elevation of production of heat which follows the ingestion of food and he recognized the fact that the intensity of the stimulus varies with different foodstuffs, being greatest with protein. The work of Lusk (a pupil of Rubner) forms the foundation of present knowledge of the nature of the specific dynamic action of the various foodstuffs. One of the earliest, and seemingly probable, theories to explain the marked increase in production of heat which follows the ingestion of food was that advanced by von Mering and Zuntz (9) who believed that the increased activity of the gastro-intestinal tract would account for the increase in the consumption of oxygen. Rubner (9), however, showed that the ingestion of bones by a dog failed to raise the level of metabolism. Following this work, however, several papers appeared which seemed to support the theory of von Mering and Zuntz, but it remained for Benedict and Emmes to show conclusively that even violent peristalsis in the gastro-intestinal tract, such as followed the ingestion of sodium sulphate and large amounts of agar agar, caused hardly any change in the level of consumption of oxygen or the production of carbon dioxide in man. Increased secretory activity of the various glands of the gastro-intestinal tract was likewise eliminated as a factor by the

experiments of Rubner and later of Lusk (7) who showed that the ingestion of Liebig's extract of beef, although causing a flow of gastric and pancreatic juices, was without effect on the level of the consumption of oxygen. Other purely mechanical changes brought about by the ingestion of food which might theoretically be considered capable of elevating the level of metabolism, such as changes in osmotic pressure of the blood and increased renal activity, were eliminated by Lusk (6) when he demonstrated that the ingestion of urea in quantities which would be eliminated from considerable amounts of meat, and also of solutions of sodium chloride, was without appreciable effect on the basal level of the production of heat. Numerous unpublished experiments in our laboratory have confirmed these results with Liebig's extract and have likewise shown that water and physiologic solution of sodium chloride may be administered intravenously without causing any significant changes in metabolism.

It is therefore obvious that none of the commonly known physical changes which might be induced in the body by the ingestion of food could possibly account for the marked elevation of metabolism, and the explanation must be sought for in the numerous chemical interactions which take place between the living protoplasm and the ingested foodstuff. The classical investigations of Lusk have clearly demonstrated that there are fundamental differences in the manner of production of the specific dynamic action of protein on the one hand and of fat and carbohydrate on the other. Rubner (10) believed that when protein was ingested part of the protein molecule which could form glucose was used by the cells without elevating their fundamental rate of production of heat, because the glucose originating from the ingested protein simply replaced an equivalent quantity of glucose which would have been oxidized during this period; those portions of the protein molecule which could not form glucose were oxidized with the liberation of free heat, which constituted the "extra" heat of the specific dynamic action of protein. In attempting to substantiate this theory, Lusk compared the effect of administering the amino acids, alanine, glycocoll, and glutamic acid to dogs. According to Rubner's hypothesis alanine and glycocoll should exert hardly any specific dynamic action, since they had been shown by Lusk to be completely converted to glucose and urea; glutamic acid, on the other hand, had been shown to yield only three of its five carbon atoms as glucose and should, therefore, exert specific dynamic action. Actual experiment showed the reverse to be true, that is, alanine and glycocoll caused a marked increase in the production of heat, while glutamic acid was without effect. Later studies by Lusk (8) showed that alanine and glycocoll exert a marked specific dynamic action in the completely phlorizinized animal when the entire energy content of the ingested amino acid is eliminated in the urine as urea and glucose. From this he concluded: "Both alanine and glycocoll, even when

they are not oxidized and when their energy passes from the phlorizinized animal in the form of sugar and urea, yield products of metabolism, either oxy- or keto-acids, which act as stimuli to induce higher oxidation in that organism. Thus is the conclusive proof of a true chemical stimulation of protoplasm within the mammalian organism. It explains the specific dynamic action of protein." In formulating his theory of stimulation by amino acid, Lusk was careful to emphasize that the process of deaminization was not the factor responsible for the increase in heat production, since glutamic and aspartic acids were deaminized and their nitrogen eliminated as urea without causing any specific dynamic action.

Terroine and Bonnet have recently shown that in frogs glutamic acid exerts specific dynamic action; furthermore, they have found that all of the amino acids of the aliphatic series exert in frogs practically the same specific dynamic action which is proportional to the amount of nitrogen given but not to the total quantity of amino acid. They concluded that the specific dynamic action arises from the metabolization of the nitrogen portion of the amino acid. Their results deserve further study and consideration, but it must be emphasized that the results obtained in amphibians are not directly comparable to the results in mammals.

Rapport and Katz recently reported experiments in which they studied the consumption of oxygen of the isolated hind leg of a dog while it was being perfused with whole blood. In control experiments they found that the consumption of oxygen by the preparation declined steadily during the experiment. When glycocoll was added to the blood used to perfuse the leg they found that the consumption of oxygen showed a marked rise instead of the usual decline. The respiratory quotients were very irregular. From these experiments they concluded: "Glycine acts as a powerful stimulator of the cell metabolism in an isolated perfused muscle and as a corollary to the above that the specific dynamic action of glycine is a direct effect upon the cells of the tissue stimulated and that no central mechanism outside of the tissue need be postulated." In connection with their conclusions it is interesting to note that Lusk (7) at one time advanced the hypothesis that the amino acids act as direct stimuli to the tissues without undergoing chemical changes; more detailed study, however, failed to substantiate this hypothesis and it was discarded by Lusk.

Wilhelmj and Bollman have shown that when amino acids are administered intravenously to normal, well nourished dogs, there is prompt and marked elevation in production of heat which usually reaches its maximum during the ten minutes occupied by the injection and may require from four to nine hours to return to the basal level. They also demonstrated that when the specific dynamic action was expressed as calories of extra heat for each millimole of amino acid deaminized, the value was quite uniform for the same amino acid, even when given in different quantities

to different dogs, but was apparently somewhat different for different amino acids. The values obtained for alanine and glycocoll were of about the same order of magnitude, being 0.35 and 0.45 calorie, respectively, for each millimole of amino acid deaminized, while the average value for phenylalanine was about double this value or 0.70 calorie for each millimole deaminized. When these values are compared with the values obtained by Lusk for alanine and glycocoll and by Rapport and Beard for phenylalanine it is found that they are of approximately the same order of magnitude, in spite of the fact that these investigators administered the substances orally. The experiments of Wilhelmj and Bollman conclusively demonstrated two important points: that the intravenous administration of amino acids brings about a prompt and marked elevation in production of heat, and that the extra heat produced is proportional to the amount of amino acid deaminized.

It has been shown by Bollman, Mann and Magath (4) that the production of urea in the dog is entirely dependent on the presence of the liver, since the formation of urea ceases as soon as the liver is removed. The same investigators (5) have also shown that when amino acids are injected intravenously into hepatectomized animals, the total quantity injected can be recovered in the urine, blood and muscle of the animal; in other words, the injected amino acids are apparently unchanged in the body of the completely hepatectomized animal. The hepatectomized animal, therefore, offers a unique opportunity for study of the effect of intravenously administered amino acids on production of heat when the organism is unable to deaminize the injected amino acid.

Mann, Wilhelmj and Bollman recently reported experiments in which they studied the effect of intravenously administered amino acids on the respiratory metabolism of hepatectomized animals and failed to obtain evidence of specific dynamic action. These experiments, however, were not entirely conclusive because the level of consumption of oxygen after hepatectomy and before the injection of amino acid was markedly elevated above the true pre-operative basal value. Under these conditions we believed that the specific dynamic action of the amino acids might possibly be masked by the abnormally high metabolism. The experiments reported in this study constitute an entirely new series in which this objection has been successfully eliminated.

METHODS. The liver was removed by the three-stage technic developed by Mann. Animals on which the two preliminary operations (the establishment of a reverse Eck fistula with ligation of the vena cava above the anastomosis, and ligation of the portal vein) had been performed several months previously, were placed on the standard diet used in the laboratory (17) and carefully trained for metabolic studies. During the training period the basal level of production of heat, eighteen to twenty hours after

the last feeding, was determined almost daily for long periods, until the true basal level had been reached, and was constant from day to day. After the animals were thoroughly trained the specific dynamic action of the amino acids, alanine and glycocoll, and also of glucose, were determined several times on each animal according to the technic previously described (17). A large number of control experiments were also performed in which the animals were deeply anesthetized with ether for ten to twenty minutes and the specific dynamic action of the amino acids determined at various intervals after the anesthetic had been discontinued.

The animals were fasted twenty-four and sometimes forty-eight hours before hepatectomy was performed. The general technic for determining the respiratory metabolism following hepatectomy has been described by Mann and Boothby. In the morning before the operation a series of four to six satisfactory determinations of basal production of heat was made. The liver was then removed under ether anesthesia, the average time required for the complete operation being about twelve minutes, while the period of anesthetization averaged about twenty minutes. Following operation the animals were permitted to recover completely from the effects of the anesthetic, which usually required from one and a half to two and a half hours. Following this, two or more satisfactory tests of ten minutes' duration were obtained after which the solution of amino acid was injected intravenously. The time required for injection was always ten minutes and the respiratory metabolism was determined during injection. Following injection, tests of ten minutes' duration, with three-minute intervals between tests, were obtained for several hours, during which time the effect of glucose and of further injections of amino acid was frequently determined.

In all satisfactory experiments the animals appeared normal following the metabolic experiments and frequently other experiments were performed at the end of the respiratory experiment.

Alanine and glycocoll were the only amino acids used in these experiments. The standard dose employed was 0.1 gram of amino acid nitrogen for each kilogram of body weight (pre-operative). This amount of amino acid was dissolved in 50 or 60 cc. of distilled water and warmed approximately to body temperature before injection. Glucose was administered as a 25 per cent aqueous solution, in amounts of 0.25 to 0.5 gram for each kilogram of body weight.

RESULTS. Figures 1 to 7 show the results obtained in these experiments. A large series of experiments showed that animals in which the two operations preliminary to removal of the liver had been performed several months previously, responded in a perfectly normal manner to the intravenous injection of amino acids (figs. 4 and 6). Before the liver was removed, consumption of oxygen showed a prompt and marked increase,

possibly reaching its height during the injection of the amino acid or within a few minutes afterward. The respiratory quotient was likewise promptly elevated. In several of the animals the urine was collected during the respiratory experiment and it was found that the rate and quantity of deaminization were practically the same as in normal animals not operated on. The specific dynamic action expressed as calories for each millimole of amino acid deaminized was likewise practically always within the limits found in normal animals. In animals on which the two operations preliminary to hepatectomy had been performed, the liver was usually definitely atrophied but the remaining tissue was apparently functionally adequate. One clear-cut exception to this general statement was noted in an animal in which the liver was unusually atrophic and in which the specific dynamic action of amino acids, although not absent, was definitely reduced.

Since hepatectomy was to be performed under ether anesthesia, a series of control experiments was carried out in which the animal was kept under deep ether anesthesia for ten to twenty minutes and the effect of the injection of amino acid determined at varying intervals after recovery. It was found that during the first hour after anesthesia was discontinued the metabolism was usually, but not invariably, markedly elevated. This was apparently the result of a true increase of basal metabolism since it occurred even when the behavior of the animal, as regards movements, was perfect. Injection of amino acids during this period of high metabolism either failed to show any further increase in metabolism, or else the increase was slight and transient (fig. 1). From one and a half to two and a half hours after anesthesia was discontinued the metabolism was practically always within normal limits. The injection of amino acids would now bring about the usual rapid elevation of metabolism (fig. 2). It was thereby shown that conclusive results be could obtained only when the level of consumption of oxygen after hepatectomy was practically identical with the basal preoperative level. In this connection it may be noted that Aub, Everett and Fine have also shown that the anesthetics, urethane and paraldehyde, practically abolish the specific dynamic action of amino acids administered intravenously.

One other complicating factor might possibly mask the specific dynamic action of the amino acids and lead to erroneous conclusions, namely, the specific dynamic action of glucose. Mann and Boothby have called attention to the fact that the specific dynamic action of glucose is considerably greater after removal of the liver than before, and our results have confirmed this. It is possible by repeated intravenous injections of glucose (0.25 gram for each kilogram each hour) to bring about a definite and persistent elevation of the level of consumption of oxygen in the hepatectomized animal, and it seemed entirely possible that this increase due to glucose

might mask the effect of the amino acid, although Lusk (8) has shown that in the normal animal there is almost complete summation of the effects of glucose and the amino acids, alanine and glycocoll. Because of this possible source of error in the hepatectomized animal we have always endeavored to give an injection of amino acid before administering glucose and before the animal showed symptoms of hypoglycemia.

Following hepatectomy there is a spontaneous rise in the respiratory quotient, the significance of which has been discussed by Mann and

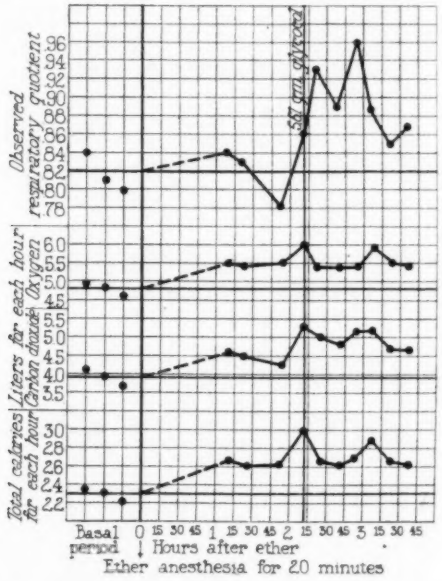

Fig. 1. (Dog 1) Weight of dog, 10.5 kgm. Ether control, September 22, 1927. The elevation of metabolism which occurs before complete recovery from ether and the transient specific dynamic action of glycocoll when given during this period.

Boothby. Following the injection of amino acids, however, there is a further rise, which is of about the same magnitude as the rise which occurs in normal animals. The calorific value of 1 liter of oxygen, calculated in the usual way, is therefore slightly higher after the injection of amino acid, and this accounts for the occasional slight rise in the total calories following the injection of amino acid in the hepatectomized animal. Since it is doubtful whether the usual manner of calculating the total calories (on the basis of the respiratory quotient) should be applied to the hepatectomized animal, it is possible that the consumption of oxygen, and not the total calories, should form the basis of comparison in these experiments.

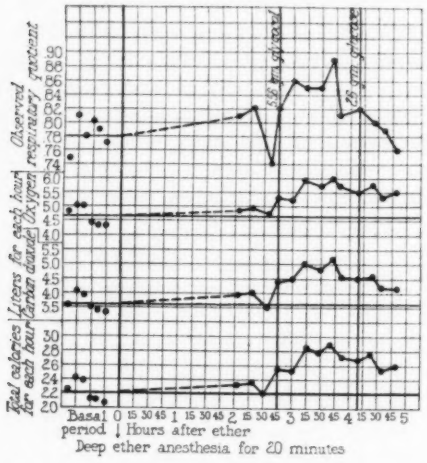

Fig. 2. (Dog 1) Weight of dog, 10.4 kgm. Ether control, September 23, 1927. The metabolism is within normal limits after complete recovery from ether and the specific dynamic action of glycocoll is now definite.

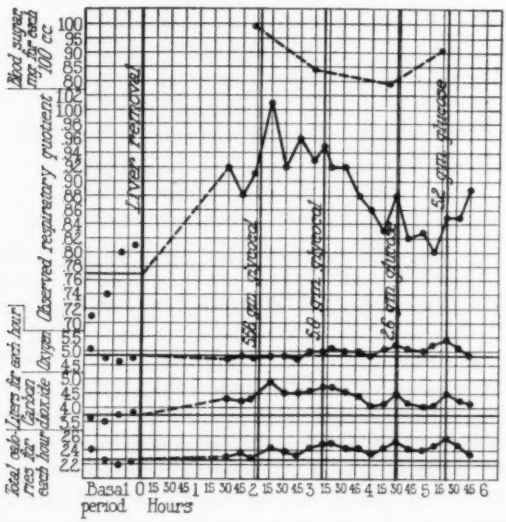

Fig. 3. (Dog 1) Weight of dog, 10.4 kgm. Hepatectomy, September 27, 1927. The specific dynamic action of glycocoll is absent after hepatectomy even when the blood sugar is within normal limits. Small doses of glucose give a specific dynamic action.

The experiments reported here show the results obtained in hepatectomized animals when all of the sources of error mentioned had been eliminated and the proper conditions fulfilled. In all the experiments the consumption of oxygen after hepatectomy, and just before the injection of amino acid, was practically the same as the pre-operative basal value. During and following the injection of the amino acid the level of consumption of oxygen remained unchanged; the small fluctuations noted are within the limits of experimental error (figs. 3, 5 and 7). If the same quantity of amino acid was given to the same animals before hepatectomy, a definite

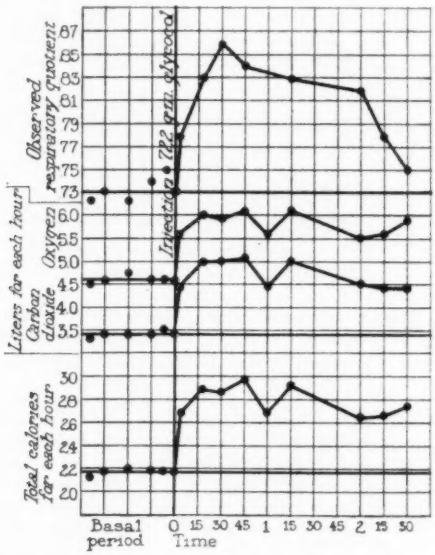

Fig. 4. (Dog 2) Weight of dog, 13.6 kgm. May 10, 1927. The definite specific dynamic action of glycocoll in an animal which had had the two operations preliminary to hepatectomy.

and prompt rise in oxygen consumption resulted in every case (figs. 2, 4 and 6).

In the hepatectomized animals there was a definite rise in the level of consumption of oxygen following the intravenous injection of 0.25 gram of glucose for each kilogram of body weight. This amount of glucose, when given to a normal animal, following a fast of eighteen to twenty hours, is practically without effect on the consumption of oxygen. This establishes the important point that the specific dynamic action of glucose is independent of hepatic function. The positive response to glucose also serves as an excellent check on the failure to respond to the amino acids, since it

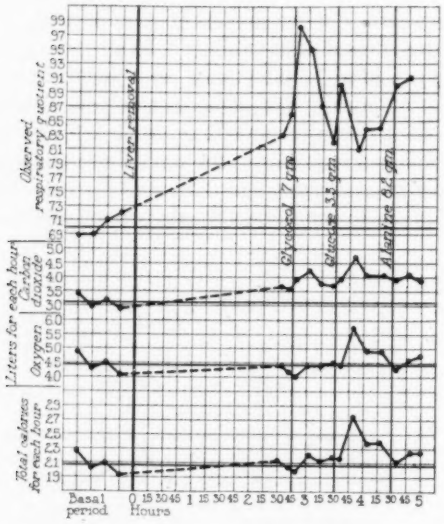

Fig. 5. (Dog 2) Weight of dog, 12.9 kgm. Hepatectomy, May 19, 1927. Absence of specific dynamic action of glycocoll and alanine after hepatectomy with marked effect from 0.25 gram of glucose for each kilogram of body weight.

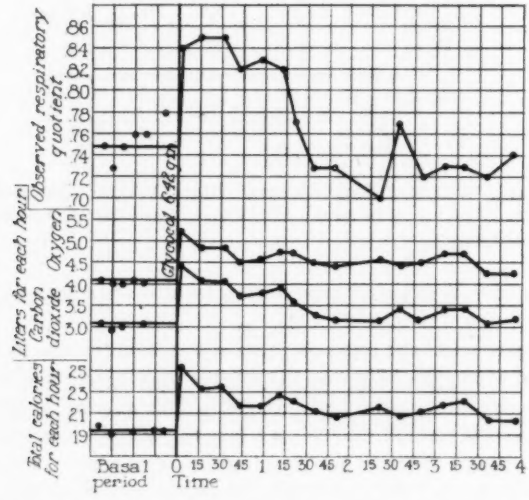

Fig. 6. (Dog 3) Weight of dog, 12.0 kgm. November 2, 1927. Marked specific dynamic action of glycocoll in an animal which had had the two operations preliminary to hepatectomy.

shows that the animals were physiologically capable of responding to a stimulus which was not dependent on the liver.

COMMENT. Following total removal of the liver in the dog, the specific dynamic action of intravenously administered amino acids is either abolished or decreased to within the limits of experimental variation. This conclusion is based on a series of ten experiments in which there were no exceptions to this result. Aub and Means believed that the liver played an important part in the specific dynamic action of protein but could not demonstrate that it was decreased in clinical cases of severe cirrhosis and of gallstones. They concluded: "That either the liver is not the main site

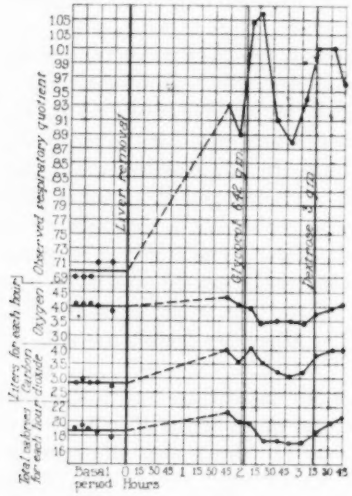

Fig. 7. (Dog 3) Weight of dog, 12.0 kgm. Hepatectomy, November 10, 1927. Absence of specific dynamic action of glycocoll following hepatectomy.

of the specific dynamic action of protein, or that it can adequately perform that function even in disease." From the results we obtained in dogs on which the two operations had been performed preliminary to removal of the liver and in which the liver was markedly atrophied, it is likely that a large part of the liver could be destroyed without definitely influencing the magnitude of the specific dynamic action of protein.

As an explanation of our results it seemed possible that the blood sugar was probably low at the time the amino acids were injected and that in the absence of normal concentration of blood sugar the characteristic reaction was prevented. In attempting to verify this hypothesis, several experiments were performed in which glucose (0.25 gram for each kilogram of body weight) was injected shortly before the amino acid, but even under

such conditions the results were uniformly negative. In one experiment the blood sugar was determined at frequent intervals; just before the first injection of glycocoll the blood sugar was 0.099 per cent; one hour and five minutes after the first injection of glycocoll and ten minutes before the second, the blood sugar was 0.084 per cent; one hour and ten minutes after the second injection it had only dropped to 0.079 per cent (fig. 3). Here a normal level of blood sugar existed at the time of both injections of glycocoll, and remained within normal limits for slightly more than an hour after each injection, but evidence of specific dynamic action was not present. From these results it is clear that the absence of specific dynamic action of amino acids in hepatectomized animals is not dependent on reduced concentration of blood sugar.

The rise of the respiratory quotient which follows the injection of amino acids in hepatectomized dogs is of about the same magnitude as that found in normal animals (17). Wilhelmj and Bollman suggested three possible explanations for this elevation of the respiratoryquotients: 1, that it is the result of disturbance in acid-base equilibrium with liberation of carbon dioxide; 2, that it might result from the combustion of glucose derived from the amino acid per se, and 3, that it represents true stimulation of utilization of carbohydrate. The fact that it occurs in hepatectomized animals definitely eliminates the second possibility from further consideration since it has been shown that amino acids are unable to form glucose after hepatectomy. The other two factors are still possible causes of the elevation.

The interpretation of our data brings up two important points regarding the site of production and cause of the specific dynamic action of amino acids: 1. Our findings do not prove that the specific dynamic action of amino acids normally results from the process of deaminization per se. The experiments of Lusk, in which it was shown that glutamic acid does not exert specific dynamic action (a fact recently verified by Aub, Everett and Fine using intravenous administration), in spite of the fact that it was deaminized and the nitrogen eliminated as urea, are definite evidence that the process of deamination is not accompanied by an increase in production of heat. 2. It is not likely that the increased production of heat which normally follows the ingestion of amino acids takes place solely within the liver; it is much more probable that substances formed in the liver as a result of the deaminization of the amino acids act by increasing the rate of oxidation in various tissues throughout the body.

SUMMARY

The specific dynamic action of amino acids administered intravenously is within normal limits in dogs that had had the two operations preliminary

to removal of the liver (reverse Eck fistula with ligation of the vena cava and later ligation of the portal vein). Following removal of the liver the level of oxygen consumption is practically unchanged, provided that sufficient time is allowed for the animal to recover from the ether anesthesia completely, which usually requires about one and a half to two and a half hours. Following hepatectomy the respiratory quotient shows spontaneous and persistent elevation.

The intravenous injection of the amino acids, alanine and glycocoll, into the hepatectomized animal fails to produce elevation in the level of consumption of oxygen but does produce further elevation of the respiratory quotient; the occasional slight increase in total calories, due to the increase in the calorific value of oxygen resulting from the elevation of the respiratory quotient, is within the limits of experimental variation. The absence of the specific dynamic action of amino acids in the hepatectomized animal is not the result of a low level of blood sugar, since the same result is obtained when the blood sugar is well within normal limits.

These experiments suggest that the specific dynamic action of amino acids is not the result of direct stimulation brought about by the presence of unchanged amino acids in the tissues.

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DIFFERENCES IN THE BILE FROM THE TWO SIDES OF THE LIVER

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In a previous communication (1) we described how individual lobes of the liver receive portal blood from different visceral sources. By transillumination of the portal vein and dye injections we were able to visualize the intra-portal currents or "streamlines" that determine this selective distribution of portal blood in the dog's liver. It was found that blood from the spleen, stomach, and greater part of the colon was conveyed to the left half of the dog's liver; whereas, blood from the pancreas, duodenum and jejunum passed mainly to the right side.

In the light of the above findings, experiments were carried out to determine whether there were corresponding differences in the character of the bile from the two sides of the liver. As far as we are aware the only investigations that have touched upon this study are those of Rous and McMaster (2) who, in the course of experiments on the concentrating activity of the gall bladder, determined the pigment content of bile from different portions of the dog's liver. These workers, from experiments on six dogs, concluded that "the bile coming at one time from different portions of the liver of the dog has nearly the same amount of pigment per cubic centimeter."

In this paper we present the results of our investigations on the relative output, physical characters, pigment, ash and total solids in the normal bile from the right and left sides of the liver of the dog.

METHOD. Bile was collected under aseptic conditions in rubber balloons that were left inside the abdomen. The collection of bile was continued over a period of 48 hours when the animals were killed. The dogs were fasted before and after operation.

Anatomical and technical details. The great variation in size and disposition of the hepatic ducts of the dog renders bile collection difficult. The type of duct formation depicted in figure 1 is present in about one out of 6 dogs; as this type was most accessible for cannulation and collection of bile from the right and left sides of the liver, only dogs with such a duct system were employed in our experiments.

The surgical operations were performed with the animal under ether anesthesia. Careful haemostasis was made and the tissues were handled with minimum trauma. The cystic duct, *c*, was ligated close to its termination so as to exclude the gall bladder from communication with the duct system. The duct, *lh*, in the dogs used in our experiments was found by dissection and subsequent injection of trypan blue to drain bile from the two largest lobes of the left side of the liver (i.e., the whole of the left half of the liver except the small lobule immediately to the left of the gall bladder). In a few instances the tributary duct, *d*, joined its fellow from the left side very close to the main hepatic duct. In these cases (2) it was

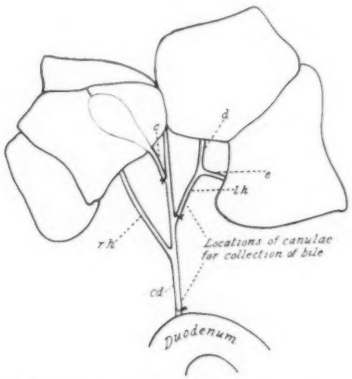

Fig. 1. Diagrammatic drawing showing the type of distribution of hepatic ducts that was used in our experiments for collection of bile from different portions of the dog's liver. *c*, cystic duct; *c.d.*, common duct; *d.* and *e.*, hepatic ducts that unite to form *lh.*, left hepatic duct; *rh.*, right hepatic duct. Ligatures mark the sites of ligation of the cystic, left hepatic and common bile ducts. A cannula was placed proximal to the ligature about the common duct for collection of bile from the right side of the liver. Another cannula was inserted into the left hepatic duct beyond the ligature about it for collection of bile from the left side of the liver.

only possible to collect bile from the duct *e* that drains the large lobe on the extreme left of the liver. Reference is made to this difficulty in table 1.

In all instances the duct *lh* was ligated near its termination and a slit was made in its wall to allow the entry of a glass cannula with a fairly long shank. Bile was collected from the rest of the liver by the insertion of a cannula into the common bile duct just above its termination in the duodenum.

The rubber collecting bags that were attached to the cannulae were always completely deflated and were placed so that they lay snugly in the abdominal cavity without causing any drag on the ducts or adjacent

structures. The abdominal walls were carefully approximated by layers of sutures. The operations were well tolerated.

At the end of the experiments the collecting bags were removed from the abdomen without displacing the cannulae from the ducts. The liver was removed and the ducts were dissected carefully so as to ascertain the portion of the liver that they had drained. Confirmation of the area of drainage of the ducts was obtained by the injection of trypan blue into the ducts through the cannulae that had been left in situ. The portion of liver stained by the dye represented the area of liver that had been drained by

Comparisons of bile collected under identical con

	DOG 1		DOG 2		DOG 3		DOG 4		DOG 5	
	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left
Weight of liver tissue, grams...			290	160	145	111	140	290	143	245
Color.....	Deep brown	Green-ish	Dark brown black	Grass green	Dark black brown	Grass green	Very dark brown	Green-ish	Dark brown	Green-ish
Volume of bile:										
a. Cubic centimeter total collection.....	56	10	37	12	33	17	90	32	54	8
b. Cubic centimeter per gram tissue.....			0.127	0.075	0.228	0.154	0.643	0.110	0.378	0.032
Specific gravity.....	1.01975	1.04302	1.02140	1.02636	1.01494	1.02090	1.01279	1.01530	1.01803	1.027
Total solids:										
a. Parts per 1000 by weight..	60.8	115.8	65.1	89.2	49.6	74.3	41.2	50.7	67.0	68.6
b. Grams total excretion.....	3.47	1.62	2.46	1.09	1.66	1.29	3.75	1.64	3.68	0.56
c. Milligrams per gram liver..			8.48	6.86	11.4	11.6	26.4	5.65	25.9	2.28
Ash:										
a. Parts per 1000 by weight..	5.75	11.1	6.45	6.75	5.12	5.25	5.45	6.05	5.55	6.50
b. Grams total excretion.....	0.325	0.115	0.244	0.083	0.171	0.091	0.501	0.196	0.302	0.053
c. Milligrams per gram liver..			0.841	0.519	1.18	0.819	3.57	0.676	2.09	0.216
Ash ..... Total solids	9.2%	7.1%	9.8%	7.5%	10.3%	7.07%	13.3%	11.9%	8.2%	10.3%
Pigments:										
a. Milligrams per cubic centimeter.....	0.845	1.89	0.963	1.07	±0.172	0.344	0.240	0.256	±0.092	0.770
b. Milligrams total excretion.	47.3	18.9	35.5	12.8	±5.61	5.78	20.7	8.19	±4.97	6.32
c. Milligrams per gram liver..			0.123	0.080	±0.039	0.052	0.148	0.028	±0.035	0.028

one of the cannulae; the left side was the one usually selected for dye injection. The two masses of liver tissue from which bile had been drained were weighed carefully. The areas of the right and left sides of the liver from which bile was collected separately in dog 1 are shown in figure 2.

ANALYTICAL PROCEDURE. Samples of bile were emptied from the collecting balloons into sterile bottles which were at once put into a temperature equilibrating chamber where they attained a temperature of 25°C. before portions were drawn into a pycnometer for specific gravity determination.

Bile pigments were determined by the method of Hooper and Whipple

(3), 1 cc. of bile being made up with the acid alcohol reagent to a volume of 50 cc., 100 cc. or 200 cc., according to the pigment concentration. When the color allowed these were read against a standard of pure bilirubin which had been prepared from ox gall stones according to the method of Kuster (4) and dissolved in chloroform in a concentration of 0.25 mgm. per cubic centimeter. The colors developed in the acid alcohol ranged from blue to blue green so that in many cases they were hard to match against the bilirubin standard. To provide for this a 10 per cent solution of copper sulphate was evaluated against the bilirubin standard and by the addition of from one to

entical con right and the left sides of the liver of dogs

	DOG 6		DOG 7		DOG 8		DOG 9		DOG 10		DOG 11	
ft	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left
	143 Dark brown	245 Green-ish	110 Brown	140 Brown	155 Light brown	131 Light brown	318 Brown	110 Brown	132 Brown	94 Green-ish brown	Brown	Greenish brown
	54	8	43	12	15	5	128	28	57	17	32	6
0	0.378	0.032	0.391	0.085	0.096	0.038	0.402	0.254	0.431	0.180		
530	1.0180	1.02794	1.01436	1.02032	1.02621	1.01716	1.01300	1.01350	1.01293	1.01335	1.01479	1.01659
	67.0	68.6	40.5	63.8	100.4	60.1	36.8	40.9	31.1	39.5	49.0	68.4
	3.68	0.56	1.76	0.78	1.55	0.31	4.77	1.16	1.79	0.68	1.59	0.42
	25.9	2.28	16.0	5.57	10.0	2.33	15.0	10.6	13.5	7.24		
	5.55	6.50	4.74	6.51	7.61	4.10	5.48	5.63	4.57	4.78	4.94	6.27
5	0.302	0.053	0.207	0.079	0.118	0.020	0.710	0.159	0.264	0.082	0.160	0.038
5	2.09	0.216	1.88	0.570	0.756	0.159	2.23	1.45	2.00	0.87		
	8.2%	10.3%	11.7%	10.2%	7.58%	6.82%	14.8%	13.7%	14.6%	12.1%	10.0%	9.2%
	±0.092	0.776	0.393	0.980	0.693	0.103	0.218	0.247	0.232	0.392	0.230	0.306
	±4.97	6.32	16.8	11.7	10.35	0.515	27.9	6.92	13.2	6.66	7.36	1.84
	±0.035	0.026	0.152	0.084	0.066	0.004	0.087	0.063	0.100	0.071		

three drops of 1 per cent potassium dichromate to the colorimeter cup containing this inorganic standard a suitable range of colors was obtained without appreciable alteration of its value. When possible the alcoholic solutions of bile from the two sides of the same liver were read against each other as a check on the comparison. Two of the bile samples produced a purple color shortly after mixing with the acid alcohol reagent. These were compared with the corresponding sample from the other side of the liver by dilution so that an approximate estimation could be made.

To determine total solids weighed samples of bile in porcelain boats were placed in a glass tube which passed through a water jacketed oven.

Through the tube a current of air dried by sulphuric acid was drawn at reduced pressure. To determine the ash larger samples of bile were first dried and then heated to a red heat in an electric muffle for three hours.

RESULTS OF EXPERIMENTS. By selecting dogs with a suitable distribution of the hepatic ducts so that they might be cannulated we have succeeded in collecting bile from portions of the liver that correspond almost to the right and the left half of the organ. Anatomically, the liver is regarded as being divided into two halves by the interlobar cleft that passes from the fossa for the gall bladder to the entrance of the hepatic veins into the vena cava. Three lobes are situated to the left of this dividing line; the lobe immediately to the left of the gall bladder is very small and the other two are large, it is from these two latter that we have collected bile, for comparison with that secreted from the rest of the liver (table 1).

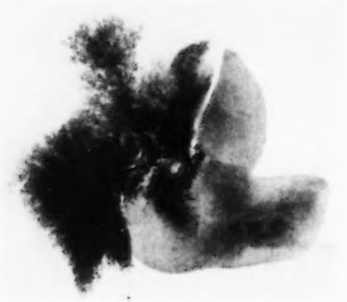

Fig. 2. Print made from x-ray film of liver of dog 1 after injection of bile ducts draining the right side of the liver with iodized oil. In this instance the areas of the right and left sides of the liver from which bile was collected separately are grossly equal in size.

In the eleven consecutive dogs from which bile was collected definite differences were observed in the color of the secretion from the two sides of the liver. In eight instances bile from the left side bore a dark green color while that from the right side was of a brown or blackish-brown tint. In the remaining 3 dogs the bile was of about the same color, generally very dark brown. In no instance was the bile unusually viscid.

With the exception of that from dog 8 the succeeding findings were consistent in all samples of bile examined. We are unable to account for the reversal of the results in the case of this dog, unless perhaps it was due to some technical error made at operation. In this animal it will be noted that only small quantities of bile were obtained from each duct. The exceptional findings in this dog are excluded from the subsequent discussion.

A comparison of the volumes of bile excreted by the right and left lobes of the liver shows that the right side produced a much more liberal excretion in contrast to the left. The greatest difference was present in dog 6 and the least in dog 3; the figures in dog 6 were 0.378 cc. per gram of liver tissue on the right side compared with 0.032 cc. on the left side, whereas in dog 3 the readings were 0.228 and 0.154 respectively.

In regard to the concentration of the bile, the results indicate that the left lobes produce a more concentrated product than the right. This is shown by the specific gravity, pigment, total solids and ash determinations. Bile that came from the left side of the liver was always of a higher specific gravity than that from the right side. Likewise, the total solids and their ash, measured in parts per 1000 by weight, reflected the constancy with which the left side of the liver elaborated bile of greater concentration than did the right side. A comparison of these two series of figures shows that the total solids in bile collected from the right side of the liver contain a greater per cent of inorganic material than found in the bile collected from the left side. The average of the ash for the series of ten dogs is 11.3 per cent of the total solids in bile obtained from the right lobes in contrast to 9.8 per cent present in the samples from the left lobes.

In the case of the pigments the same difference on the two sides obtains, the pigment concentration in the bile from the left side being considerably greater than that from the right side. The average of the series was 0.394 mgm. per cubic centimeter excreted by the right side as compared with 0.649 mgm. from the left. Rous and McMaster who, as a preliminary experiment on the concentrative activity of the gall bladder, compared samples of bile from different portions of the liver that did not belong strictly to the right and left sides, did not find this marked difference. Our variance with their findings may perhaps be accounted for by the fact that their collections of bile were not so strictly confined to the right and left sides of the liver.

It would seem that although the left lobes elaborate a bile of higher concentration, the right lobes produce a larger volume and greater weights of total solids, pigments, and bile ash per gram of liver tissue.

Discussion. All parts of the liver have been commonly regarded as having the same physiological activity. Evidence has gradually accumulated to show that this belief may not be true. It has been mentioned that previous work indicates that there is a selective distribution of the portal blood in the liver.

The physical and chemical findings in the bile collected from the right and left sides of the liver respectively indicate that there may be a varying physiological activity in different parts of the liver during the same period of time. The quantitative differences in excretion of bile by the two sides of the liver may be due to the origin of their blood supply from separate

parts of the portal system. The fact that blood coming from the small intestine passes chiefly to the right side of the liver suggests a possible relation between the larger proportion of bile solids in the total excretion from that side and a cholagogic action of bile salts absorbed from the small intestine. There is no convincing evidence at present that there is a qualitative difference in functional activity of circumscribed groups of hepatic cells.

It is interesting, in the light of our evidence of a physiological bilaterality of the liver, that McIndoe and Counsellor and others (5) have demonstrated that in the human liver an anatomical bilaterality exists. The line of cleavage between the two halves of the liver extends from the gall bladder to the entrance of the hepatic veins into the inferior vena cava. It does not correspond to the dividing line usually depicted in anatomical text-books.

SUMMARY

The relative output, physical character, pigment, ash and total solid in the bile, which was collected over a period of forty-eight hours after operation, from the right and left sides of the liver of the dog have been studied. The right side of the liver produced a greater volume of bile per gram of liver tissue than the left; however, the left lobe produced a more concentrated bile than the right lobe. The pigment concentration in the bile from the left side of the liver was considerably greater than that from the right side.

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PHYSIOLOGICAL ACTIVITY AND THE MANOILOV REACTION

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Results obtained by several investigators make it clear that the Manoilov reaction is neither a specific test for sexuality, nor for any specific sex hormone or similar substance; it is a purely quantitative test. Under certain conditions the reaction does, however, differentiate the sexes correctly in a notably high percentage of cases in species ranging from lower plants to higher animals. These results indicate that although the reaction is not a true or valid test for sex, it is somehow related to sexuality. In an earlier study (1927) we were able to show that in some particular cases where the test gave a wrong indication of the sex of a pair of animals these animals were, at the time of the test, in an unusual physiological state. More specifically, a pair of pigeons which usually gave untrue or "reversed" sex reactions at one period of the reproductive cycle (ovulation, incubation) gave correct sex reactions during other and usual life-periods.

After our studies were concluded Galwialo and co-workers (1926) published data which essentially clear up the chemistry of this reaction. They presented a list of substances which do not affect the reaction at all, and other lists of substances that do affect it in various degrees. In general, it was shown that only those compounds which are more easily oxidized than dahlia will limit or inhibit the decoloration of the dahlia used in the test. The dahlia color is rather strongly protected and preserved (female reaction) by such compounds as lecithin, creatin, protein (dialyzed egg albumen), hemoglobin; and still more strongly by phenol (and its derivatives) and tyrosin. They conclude that in work with blood and similar fluids with high protein content it is chiefly the protein that is measured by this reaction. This is doubtless true; but almost certainly it is the *total* amount of *all* substances which protect the dye from oxidation by $KMnO_4$ in acid medium that is measured.

From one standpoint it would be of much interest to learn which easily oxidizable substances exist in greater amounts in female blood, sap, or tissue extracts, in species so different as are animals and higher and lower plants. On this point we shall make reference to an apparent widespread

sex difference in the lipoid content of blood and tissues of animals and plants. It is also highly desirable to learn whether the total of readily oxidizable substances undergoes fluctuations in blood and tissue in correspondence with an increase or decrease of physiological activity. The present study, an abstract of which was previously reported (1927b), is chiefly a contribution toward a solution of this problem.

METHODS. For this study it was essential that a closely graded series of relatively permanent color standards should be prepared, since it is necessary to compare two or more samples from the same individual at rather widely separated periods of time. We found that highly satisfactory standards could be prepared from a series of dilutions of (Merck's) nicotine, to which the Manoilov reagents were added in the usual way. At one end (no. 1) of the series it is necessary to use enough nicotine (0.250 cc.) to protect completely the dahlia from any diminution in the density of its color (3 drops in 3 cc. distilled water). At the other end (no. 14) of the series, an amount is used (0.001- cc.) which just permits complete decoloration of the dye. Thus we obtained 14 color grades, nos. 1 to 14; the smaller numbers here correspond to deeper colors (so-called female reaction), and the higher ones to progressively lighter colors (male reaction). These amounts of nicotine were made up to 3.0 cc. with distilled water in small vials. The ordinary Manoilov reagents were then added to each. When these vials were sealed they were ready to use. They retained their color during several months. Precisely similar vials, containing always 3 cc. of contents, were used in making all tests of blood and tissue extracts.

TABLE 1

Summary of color grades (1–14) obtained from Manoilov tests on different organs of male and female ring doves (10 per cent aqueous extracts)

SEX	EGG YOLK*	LIVER	KIDNEY	NORMAL RIGHT TESTIS	BREAST MUSCLE		HEART	OVARY	GIZZARD
					Outer (red)	Inner (grey)			
Average male		4.5	5.0	7.7	11.5	11.5	11.55		
Number of tests		26	12	10	4	4	22		
Average female	8.7*	4.9	5.35		10.4	11.4	11.6	11.7+	14.0
Number of tests	14	40	26		15	12	33	17	2
Mean for sexes	8.7*	4.7	5.2	7.7	11.0	11.5	11.6	11.7+	14.0

* Here 5 per cent extracts were used.

The reagents used were as follows: 1. Dahlia, 3 drops of a 1 per cent alcoholic solution. 2. $KMnO_4$ 10 drops, 1 per cent (aqueous), from a pipette

which thus delivered 0.5 cc. 3. HCl, 3 drops, 40 per cent (conc. aqueous). 4. Thiosinamine, 7 drops, 2 per cent (aqueous). The vials were shaken after the addition of each of the first two reagents, and three times inverted after the addition of each of the last two reagents. In work with whole blood all samples showing coagulation were rejected. Tissues were thoroughly ground in a mortar and extracted with distilled water during approximately 4 hours.

RESULTS. *Comparison of different organs in male and female.* In this study the weight in grams of the tissue taken for extraction was considered equal in volume to exactly the same number of cubic centimeters of water; 9 times this quantity of distilled water was added for making the extraction. The results are given in table 1. The 10 per cent extracts of egg yolk were so concentrated as to leave the dahlia color too dark (nos. 1 to 3) for satisfactory readings; 5 per cent extracts of this tissue were therefore used. Taking this into account it will be seen that the egg yolk gave the lowest values (most female reaction), with liver, kidney, testis, breast muscle, heart, ovary and gizzard following in the order named. The organs stand in quite the same order whether taken from males or females. In general, our extracts from male organs (not blood) do not tend to give a higher male reaction than do the corresponding organs of the female; if there is any difference it is rather the reverse, but the fact that no attempt was made to take these samples from males and females of the same age and race makes a comparison on the basis of sex quite uncertain.

The point of chief interest in these results is that tissues which probably have a low rate or degree of activity (egg yolk, liver) are at the bottom of the series (female reaction), while those organs which are probably most active physiologically (heart, gizzard) stand at the top. The ovary occupies a very high place here, though our preconceptions of this organ would perhaps not lead us to place it high in the scale of physiological activity.

Blood from birds of different ages and with different metabolic rates. On this point a large number of tests were made. It happens, however, that proper comparisons of the effect of age can be made only for birds of the same race or strain. In the case of some races there are too few determinations to provide data of value. In table 2 we have brought together results for the five races which supply data covering an age difference of 18 months. These data rather clearly indicate that the blood of the younger birds gave lighter grades of color (male reaction) than did older birds of the same sex and strain. Incidentally, in the entire study there were also 20 groups (1 to 6 birds per group) of males paired with females of the same age and race. In 19 of these group-tests the males gave lighter color, and in 1 case darker color. Finally, 16 miscellaneous males of still younger age (3.3 months) than those shown in the table gave an average color grade of 12.8; 9 similar females (3.1 mo.), an average of 12.6.

If these tests are in fact representative of age differences shown by the Manoilov reaction, we are here able to associate the so-called "male reaction" with a higher metabolic rate; for, in unpublished studies by Benedict and Riddle, it has been shown that these young doves and pigeons have a higher basal metabolism than that of the older birds.

Tests were also made on 9 males and 6 females whose basal metabolism had been measured 1 to 3 days previously. The results, including the heat-production values (of Benedict and Riddle), are given in table 3. Meas-

TABLE 2

The relation of age to grade of color obtained from serum (0.5 cc.) by the Manoilov test

The birds compared are of same race and sex. Number of tests given in parentheses.

KIND OF BIRD	STRAIN	AGE GROUPS			
		6 to 12 months	12 to 18 months	18 to 24 months	24+ months
		color grade	*color grade*	*color grade*	*color grade*
Ring doves	1	11.5 (1)	10.7 (6)		6.0 (1)
	2	11.3 (1)	8.0 (2)	8.9 (2)	8.0 (1)
Common pigeons	3		11.8 (4)	9.4 (2)	7.0 (1)
	4		6.0 (2)	5.5 (1)	7.7 (2)
	5	7.4 (2)	6.9 (4)	6.5 (1)	

TABLE 3

Grades of color obtained from whole blood (0.1 cc.) and from serum (0.5 cc.) of male and female doves whose basal metabolism was measured

SEXES	NUMBER	BASAL METABOLISM	WHOLE BLOOD			SERUM		
			Minimum	Maximum	Average	Minimum	Maximum	Average
		*calories**	*color grade*	*color grade*	*color grade*	*color grade*	*color grade*	*color grade*
Males	9	3,532	10.5	12.4	**11.2**	12.5	13.0	**12.5**
Females	6	3,291	5.9	12.5	**8.9**	8.0	12.8	**11.0**

* Per 150 gram bird during 4 hours.

ures were made on both whole blood and serum of each bird. Considered as groups, the males gave lighter color tests (male reactions) in both blood and serum. They also showed the higher rate of heat-production.

In this connection we may describe the results of a comparison of tests made on the blood of pigeons before and after 10 minutes of enforced flight. It was at first thought that by this means the Manoilov test could be used to report the effects of physiological activity and the temporarily increased metabolism which is unquestionably associated with vigorous movement.

It developed, however, that the blood lost for the first sample was sufficient—over the short intervals that were being measured—to dilute the blood in such a way as to interfere with the reading obtained on the second sample. Actually, 8 pairs of blood samples were drawn in the following order: The *first* sample from the resting bird; the second sample taken 10 minutes later—at the conclusion of the period of flight. Of these 8 tests 7 gave the lighter color (male reaction) at the end of the exercise period; 1 gave darker color. In 4 tests of the other type the *first* sample was drawn immediately after the period of exercise, and the second sample an hour or two later. Here 2 of the tests showed lighter color and 2 darker color at the end of the period of flight. For the reason already stated these unsatisfactory tests were discontinued.

Tests on secretory glands during activity and at periods progressively removed from secretory activity. The oviduct of the pigeon provides us with

TABLE 4

Color grades obtained from each of the three parts of the oviduct at and apart from its period of secretory activity (10 per cent aqueous extracts)

PART OF OVIDUCT	FUNCTIONING, OR WITHIN 10 HOURS BEFORE FUNCTIONING	TEN TO 48 HOURS BEFORE FUNCTIONING	MORE THAN 48 HOURS FROM FUNCTIONING	WITHIN 48 HOURS AFTER FUNCTIONING
Shell gland	13.8+	13.0+	10.4	8.0
Number of tests	4	2	4	1
Isthmus	13.0	10.8+	10.5	10.5
Number of tests	1	5	2	2
Albumen gland	11.1+	9.0	8.4	5.5
Number of tests	4	3	6	1

three glands admirably adapted to study in periods of rest and activity. The tests made on these glands are summarized in table 4. The headings of the table are written in the order of their readiness to function—beginning with actual functioning. It will be observed that lightest colors (male reactions) were obtained during functional activity, and progressively darker colors (female reactions) at points more removed from secretory activity. These cases seem to provide excellent evidence that the Manoilov reaction really differentiates different degrees of physiological activity—different metabolic rates.

Tests made on extracts of live and dead embryos. Extracts from freshly killed nearly-hatched embryos have been compared with embryos of similar age left dead in their shells during 1 to 3 days. The results are given in table 5. When the data from various natural groups of individuals are compared it is found that in 3 of 4 tests the dead embryos show the

deeper color (female reaction), and in a fourth case the two are nearly equal. If we look for a correct Manoilov diagnosis of sex among these embryos it will not be found. The difference between the dead and the freshly killed is much better shown by the test than is the sex of the tested embryos (these from dissimilar races).

Reaction from the right and left testes. It is well known that there is a striking difference between the right and left ovaries of birds. This

TABLE 5

Summary of color grades obtained from extracts of freshly killed and dead (1 to 3 days) whole embryos (20 per cent aqueous extracts)

KIND OF PIGEON	SEX	ALIVE OR DEAD	NUMBER	RANGE OF AGE IN EMBRYOS	RANGE OF COLOR GRADES	AVERAGE COLOR GRADE
				days		
Ring doves	Males	Alive	4	12–15	10–11	10.8
		Dead	3	12–14	5–12	7.0
	Females	Alive	3	13–14	10–14	11.7+
		Dead	3	13–14	4.5–12	7.3
Common pigeons	Males	Alive	3	13–17	8–13	10.3
		Dead	2	14–17	7–14	10.5+
	Females	Alive	1	20	13	13
		Dead	1	19	6	6

TABLE 6

Summary of color grades from right and left testes

These also classified according to the relative size of the right and left testis (10 per cent extracts).

KIND OF PIGEON	ORDER OF TESTIS SIZE	NUMBER	RIGHT TESTIS		LEFT TESTIS		AVERAGE COLOR GRADE
			Weight	Color grade	Weight	Color grade	
			mgm.		*mgm.*		
Ring doves	Right larger	9	672	7.7	506	6.6	7.1
	Left larger	2	461	6.0	528	5.0	5.5
Common pigeons	Right larger	3	873	9.33	670	8.0	8.67
	Left larger	3	628	7.33	665	7.17	7.25

laboratory has also earlier observed many indications of differences in the behavior of the right and left testes. In normal healthy pigeons the right testis is usually the larger. In some hybrids and in disease the left testis is often the larger, though under these conditions it will usually be found that both testes are more or less reduced in size. Under conditions unfavorable to testis growth the right testis appears to lose weight faster than does the

left. It therefore seemed desirable to examine separately the members of several pairs of testes by the Manoilov reaction; and also to have this comparison include the normal cases where the right is larger than the left, as well as other cases in which this size relationship is reversed.

The results are given in table 6. When the various groups are averaged it is found that in all of the 4 possible comparisons the *left* testes yield the darker color (female reaction). Again, the right testes give a darker color when they are paired with larger left testes (and when both testes are reduced in size) than when they are members of a normal pair. The right ovary is often or usually completely suppressed in the females, and the *left* testis is somewhat less developed in normal healthy males. The right testis must here be regarded as more truly expressing testis function at its height. We observe that it yields a lighter color (more male reaction) than does the partly subdued or inhibited left testis.

Discussion. Nearly all of the facts reported here support the view that an increased physiological activity, and an increased metabolism, cause the Manoilov reaction to report a change toward maleness; inactivity or rest in the animal or tissue are conditions which favor the female reaction. This view was first advanced by us (1927) in a study of blood changes during various stages of the reproductive cycle in pigeons; the cogent evidence obtained from that source will not be reviewed here. Some additional facts concerning the tissues used in this investigation serve to reinforce this interpretation of the Manoilov reaction. Terroine and Roche (1925) have studied the rate at which atmospheric oxygen is taken up by some minced tissues (liver, kidney, muscle) of the pigeon and of a few other species. The rate of oxygen utilization was highest in the liver, next in kidney, least in muscle. It will be noted that we find the same order of protection which these organs exert against the oxidation of dahlia by oxygen supplied by the permanganate. The conditions of the two series of tests are of course quite different, but the parallel is at least notable. Again, we here find that resting oviducal tissue gives the darker dahlia color, and Riddle and Lawrence (1916) earlier observed that the resting oviduct has a higher percentage of alcohol-ether-soluble material than the functional oviduct. Finally, the fact that the blood of younger birds gives the more male reaction fits well the unpublished results of Benedict and Riddle who find that the basal metabolism of these birds decreases with age. This same relation of age to metabolism has been observed by Benedict and Talbot (1921) and others in the human, and it is probably the prevalent condition in animals and plants generally. These several tests almost uniformly confirm those earlier published by us, and again lead to the conclusion that the Manoilov reaction is a better test for metabolic level than for sex.

Alsterberg and Häkansson (1926) observed that the ovary of the mackerel always tested darker than the testis. In viewing our results

against their observation it should be noted that no large ova (yolks) of the pigeon were included in the *ovarian* tissue used by us; these larger yolks were tested separately and found to protect the dahlia more than does any other tissue. The ovary of the fish is of course mainly a mass of ova, and their tests of ovaries resulted as did our tests of egg yolk. These authors also note that at rather short intervals of time various plant tissues may give dissimilar tests. In very extensive studies, Satina and Blakeslee (1927) have shown that the most distinct differences in leaves of green plants are shown 3 to 4 weeks after flowering, and least during the flowering period. In their work with mucors the age of the culture was found of much importance; far more constant results were obtained from cultures 7 to 10 days old.

Besides the investigation of Galwialo, to which reference was made earlier, some important observations were simultaneously reported by Schmidt and Perewosskaja (1926). These workers got 90 to 95 per cent of correct tests for sex from the blood of sheep and rabbits, except in cases where the females were pregnant. In these exceptional cases, like the female doves and pigeons at ovulation as observed by us (1927), the female blood usually gave "male reactions." They also report that the blood of female sheep has a higher specific gravity and a higher protein content than has the blood of male sheep. The usual female reaction is attributed to the excess protein, and they suggested that the bloods of other animals are likewise differentiated by higher protein and greater specific gravity in the females. We do not understand how this can be asserted for many of the animals used by various workers with this test. There is no doubt of the great protecting influence exerted by the protein—particularly by the red cells; we find whole blood about 5 times more protective than serum alone (table 3). Our own suggestion is that, in studies on the blood of higher animals at least, the blood fat and lecithin have an importance which has been essentially overlooked. Excess of these substances in the blood of the birds used by us was reported by Riddle and Burns (1927), who also cited the evidence that this difference is widespread in animals.

Two questions of some importance arise from what has been learned of the Manoilov reaction: First, the identity of those substances which (under the conditions of the test) are more easily oxidized than dahlia, and which exist in female blood in greater quantity than in male blood. A fuller knowledge of these facts would doubtless contribute something toward clearing up the biochemical basis of sex difference. Whatever may be the identity of these easily oxidizable substances in the blood and tissues studied by us, our interpretation of the results includes the view that these substances exist in larger amount in the resting tissue. Second, the nature of the influence or factor which is common to the great variety of conditions which increase or decrease the strength of the reaction. For this common element is probably associated with normal sex difference.

This second point has been of chief interest in the work reported here. In this study, and in our earlier report, we identify this common influence as degree of physiological activity, or metabolic rate. If this is correct it follows that the large amount of work that has been done with the Manoilov reaction has extended considerably the evidence for the metabolic theory of sex.

SUMMARY

Blood from younger birds gives a lighter color than is obtained from older ones, and other studies show the young to have a higher basal metabolism.

Aqueous extracts of active tissues (muscle, ovary, heart, gizzard) usually yield a lighter color than tissues presumably less active (liver, egg-yolk).

The three glands of the oviduct each give lightest color when actively secreting, and progressively more color at stages more removed from active functioning.

Extracts of whole embryos give lightest color when prepared from freshly killed embryos; decidedly darker color when obtained from embryos dead 1 to 3 days.

Similar quantitative differences between the right and left testis of pigeons have been observed, and differences between the repressed and the normal testis are indicated.

These results reveal new precautions necessary for comparisons made with this test. They supply the probable reason for many failures of the test as a sex-reaction. They further support our previously published conclusion that the reaction is a better indicator of metabolic rate than of sex, and clearly suggest that its value as a sex test rests upon a primary relation that metabolism bears to sex. The numerous studies that have been made on plants and animals with this test have therefore notably extended the evidence for the metabolic theory of sex.

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